REMARKS

Applicants respectfully request reconsideration of the present application in view of the foregoing amendments and in view of the reasons that follow.

Drawings

The Examiner has not yet indicated in the Office Action Summary that the formal drawings filed with the original application papers have been accepted. Acceptance of these drawings by checking the appropriate boxes in the Office Action Summary is respectfully requested in the next communication from the Examiner. ***This is the third request for acknowledgement that the drawings are accepted.***

Rejections Under 35 USC 112, First and Second Paragraphs

Applicants traverse the examiner's findings for lack of enablement and written description of the following reasons.

For the following reasons, applicants submit that their claimed method can be implemented with any yeast, and the examiner has proffered no evidence or rationale to the contrary. It is known, for instance, that all yeast N-linked oligosaccharides contain in common a high-mannose type sugar chain structure. See Gemmill et al., *Biochimica et Biophysica Acta* 1426 (1999) 227-237; page 229, right column, lines 13-14. (Appendix I). This structure is formed by addition of mannose by OCHl (1,6-mannosyltransferase), which all yeasts possess. See Gemmill et al. at 30, description for Figure 2, lines 1-2. In addition, yeasts generally add only mannose or mannose and galactose to their glycoprotein glycans. See Gemmill et al., at 230, right column, lines 9-10. Further, yeasts and most higher eukaryotes have an evolutionally conserved N-linked oligosaccharide biosynthetic pathway, which involves the formation of a Glu3Man9GluNAc2-PP-dolichol lipid-linked precursor See Gemmill et al., at page 227, Abstract, lines 1-3. It also is known that the sugar chain moiety synthesized in the ER is same in yeasts and mammals.

In summary, it can be said that the biosynthetic pathway of the N-type sugar chain has many properties that are common to various eukaryotic species, including all yeasts. Absent

some basis for the contrary assertion, therefore, the examiner has not sustained her burden of proof in relation to the alleged case for non-enablement.

Considering the above common properties of the biosynthetic pathway of the N-type sugar chain in yeasts, it can be reasonably expected with a high probability of success by a person skilled in the art that a method for preparing a mutant yeast producing a mammalian-type glycoprotein of the present invention is applicable for not only *Saccharomyces cerevisiae*, but also for another species of yeast. By contrast, there are no reasons for supposing that the method of the present invention can be conducted only using *Saccharomyces cerevisiae*. It is well known to persons skilled in the art that yeasts have common properties as described above, therefore it does not require undue experimentation to conduct the method of the present invention using another species of yeast. In fact, after the filing of the present application, a mammalian-type glycoprotein was produced by the same method as that of the present invention using *Pichia pastorsis*. See Choi et al., *PNAS* 100 (2003), 5022-5027. (Appendix II). This serves as evidence that the present claims are enabled.

Moreover, applicants urge that the *Lilly* decision cited by the examiner is not applicable to the present claims. In *Lilly* the claims at issue were to genes, which are compositions of matter. The present invention does not claim genes per se but rather a method of "preparing a mutant yeast." In *Lilly*, the patent right concerning human insulin cDNA was judged to be invalid, since the specification only described rat insulin cDNA, the amino acid sequence of human insulin, and a general cloning method for genes. There was no evidence concerning a relationship between the structure of rat insulin cDNA and the structure of human insulin cDNA. See Written Description Guideline citing the *Lilly* case, page 62, 6th paragraph. (Appendix III).

By contrast, as mentioned above the present invention is a method. Accordingly, the present application does not claim MNN1, MNN4, and OCHIl per se or α-mannnosidase I and N-acetylglucosaminyl transferase-I. As argued in the previous response, the possibility that genes having similar functions would resemble each other in structure is high.

In view of the above , applicants urge that they were in possession of the claimed method and the specification would allow one of skill in the art to practice the invention without undue experimentation.

Claim Rejections - 35 USC 102

The examiner maintains the rejection under 35 USC 102 (b) over Chiba et al., *J. Biol. Chem.*, 273 (1998) 26298-26304, ("Chiba et al.").

As argued in the previous response, in Chiba et al., introduction of the N-acetylglucosaminyl transferase-I (GnT-I) gene into host cells is not actually conducted. In fact, a reference to "an object of our future research" made on page 26303, right column, last line of Chiba et al., conversely suggests that further research was required for co-expression of alpha-1,2-mannosidase (α-mannosidase I) and GnT-I.

Furthermore, in Chiba et al., there is no description concerning whether or not the Golgi retention signal is necessary with respect to the GnT-I gene. At the time of the published date of Chiba et al., there was no knowledge concerning the need for the targeting signal of the GnT-I. After that, Yoshida et al., *Glycobiology*, 9 (1999) 53-58 ("Yoshida et al.") (Citation C1 of IDS filed August 5, 2004, available on PAIR) reported that "[t]he expressed GnT-I was localized in all organella, . . . suggesting that the mammalian Golgi retention signal of GnT-I did not function in yeast cells. Analysis of the GnT-I gene product . . . , the N-terminal region including GnT-I, including the mammalian Golgi retention signal, should be removed in the yeast ER." Yoshida et al., at 53, left column, abstract, lines 8 to last line from the bottom. Therefore, at the time of filing the present application, it was common knowledge for a person skilled in the art to introduce the GnT-I gene without the targeting signal. Contrary to such common knowledge, the GnT-I in a form having the targeting signal is expressed in the yeast in the present application.

Considering the above background to the views concerning the targeting signal of the GnT-I, Chiba et al. does not describe expressly or inherently GnT-I having the signal (intact GnT-I). Therefore, the present application should not be rejected as being anticipated by Chiba et al. under 35 USC 102(b).

CONCLUSION

The Commissioner is hereby authorized to charge any additional fees which may be required regarding this application under 37 C.F.R. §§ 1.16-1.17, or credit any overpayment, to Deposit Account No. 19-0741. Should no proper payment be enclosed herewith, as by a

check or credit card payment form being in the wrong amount, unsigned, post-dated, otherwise improper or informal or even entirely missing, the Commissioner is authorized to charge the unpaid amount to Deposit Account No. 19-0741. If any extensions of time are needed for timely acceptance of papers submitted herewith, Applicant hereby petitions for such extension under 37 C.F.R. §1.136 and authorize payment of any such extensions fees to Deposit Account No. 19-0741.

Respectfully submitted,

Date January 29, 2007

By _____

FOLEY & LARDNER LLP
Washington Harbour
3000 K Street, N.W., Suite 500
Washington, D.C. 20007-5143
Telephone: (202) 672-5300
Facsimile: (202) 672-5399

Matthew E. Mulkeen
Attorney for Applicants
Registration No. 44,250

APPENDIX I

ELSEVIER

Biochimica et Biophysica Acta 1426 (1999) 227–237

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

Review

Overview of *N*- and *O*-linked oligosaccharide structures found in various yeast species

Trent R. Gemmill, Robert B. Trimble *

Wadsworth Center C-547, New York State Department of Health, P.O. Box 509, Albany, NY 12201-0509, USA

Accepted 20 August 1998

Abstract

Yeast and most higher eukaryotes utilize an evolutionarily conserved *N*-linked oligosaccharide biosynthetic pathway that involves the formation of a $Glc_3Man_9GlcNAc_2$-PP-dolichol lipid-linked precursor, the glycan portion of which is co-translationally transferred in the endoplasmic reticulum (ER) to suitable Asn residues on nascent polypeptides. Subsequently, ER processing glycohydrolases remove the three glucoses and, with the exception of *Schizosaccharomyces pombe*, a single, specific mannose residue. Processing sugar transferases in the Golgi lead to the formation of core-sized structures ($Hex_{<15}GlcNac_2$) as well as cores with an extended poly-α1,6-Man 'backbone' that is derivatized with various carbohydrate side chains in a species-specific manner ($Hex_{50-200}GlnNAc_2$). In some cases these are short α1,2-linked Man chains with (*Saccharomyces cerevisiae*) or without (*Pichia pastoris*) α1,3-Man caps, while in other yeast (*S. pombe*), the side chains are α1,2-linked Gal, some of which are capped with β-1,3-linked pyruvylated Gal residues. Charged groups are also found in *S. cerevisiae* and *P. pastoris* *N*-glycans in the form of mannose phosphate diesters. Some pathogenic yeast (*Candida albicans*) add poly-β1,2-Man extension through a phosphate diester to their *N*-glycans, which appears involved in virulence. *O*-Linked glycan synthesis in yeast, unlike in animal cells where it is initiated in the Golgi using nucleotide sugars, begins in the ER by addition of a single mannose from Man-P-dolichol to selected Ser/Thr residues in newly made proteins. Once transported to the Golgi, sugar transferases add one (*C. albicans*) or more (*P. pastoris*) α1,2-linked mannose that may be capped with one or two α1,3-linked mannoses (*S. cerevisiae*). *S. pombe* is somewhat unique in that it synthesizes a family of mixed *O*-glycans with additional α1,2-linked Man and α1,2- and 1,3-linked Gal residues.

Keywords: Yeast glycoprotein; *N*-Glycan; *O*-Glycan; Oligosaccharide structure; NMR spectroscopy; Secretion

Contents

* Corresponding author. Fax: +1 (518) 473-2900; E-mail: robert.trimble@wadsworth.org

0304-4165/99/$ – see front matter
PII: S0304-4165(98)00126-3

1. Introduction

Yeasts have proven to be a good model system for the study of glycoprotein processing. Many of the secretory processes are conserved between yeasts and mammals, and, in fact, many of these components have been found in mammals by homology with the yeast counterparts [1,2]. Yeasts secrete and process glycoproteins in much the same way that mammalian cells do. They add *N*- and *O*-linked glycans to their glycoproteins, and, in general, much of the early processing of *N*-glycans is conserved between yeasts and mammals. Proteins are passed into the endoplasmic reticulum (ER) cotranslationally, glycosylated, sent to the Golgi for further processing, and then are either targeted to various organelles, become plasma membrane components, or are secreted into the periplasm. As discussed in Chapter 2, yeasts manufacture an outer wall, mainly composed of glycoprotein and polyglucose polymers, which serves as a porous barrier to retain most of the secreted glycoproteins within the periplasmic space. Many temperature sensitive mutants have been produced in *Saccharomyces cerevisiae* which block secretory transport at various steps along the pathway. These *sec*$^-$ mutants have allowed dissection of secretory processing events, and by homology, many comparable processing steps in mammalian cells.

Yeasts have been considered for the production of glycoprotein biopharmaceuticals [3–7]. They are easy and inexpensive to grow and can produce large amounts of secreted glycoproteins. Given the difficulties involved in gaining approval for glycoprotein biopharmaceuticals produced in mammalian cell lines [8], the difference between fungal and mammalian glycoprotein glycan structures, as discussed below, is a major stumbling block to the use of yeasts as a production vector [7]. Some of these structures are known to be antigenic [9,10], or to have immunosuppressive or lymphoproliferative effects [11] in humans. Specific yeast mannans will bind certain serum proteins such as amyloid P component [12], mannan binding protein [13], interleukin 2 [14], or CSL [14]. Other structures, similar in size and composition to mammalian high-mannose oligosaccharides, have not been sufficiently studied to determine what, if any, adverse effects they may have in humans.

Yeast glycoprotein glycans have terminal galactose and/or mannose which would likely cause the yeast-derived glycoproteins to be rapidly removed from circulation by the asialoglycoprotein receptor in the liver, and the macrophage mannose receptor, respectively. These properties may be advantageous, however, when targeting a glycopharmaceutical to these cell types is required. For example, certain high-mannose-type oligosaccharides on recombinant glycoprotein vaccines expressed in yeast have been proposed to be beneficial for delivery to antigen presenting cells [3], and yeast mannans have been proposed as vectors to deliver antibiotics to macrophages [15]. Determining the safety and efficacy of yeast-derived biopharmaceuticals, however, will first require learning the exact structures produced by the expressing organism and any undesirable effects unusual glycan structures may have in humans.

2. General considerations

2.1. N-Glycosylation

As discussed in Chapter 3, *N*-glycan precursors are

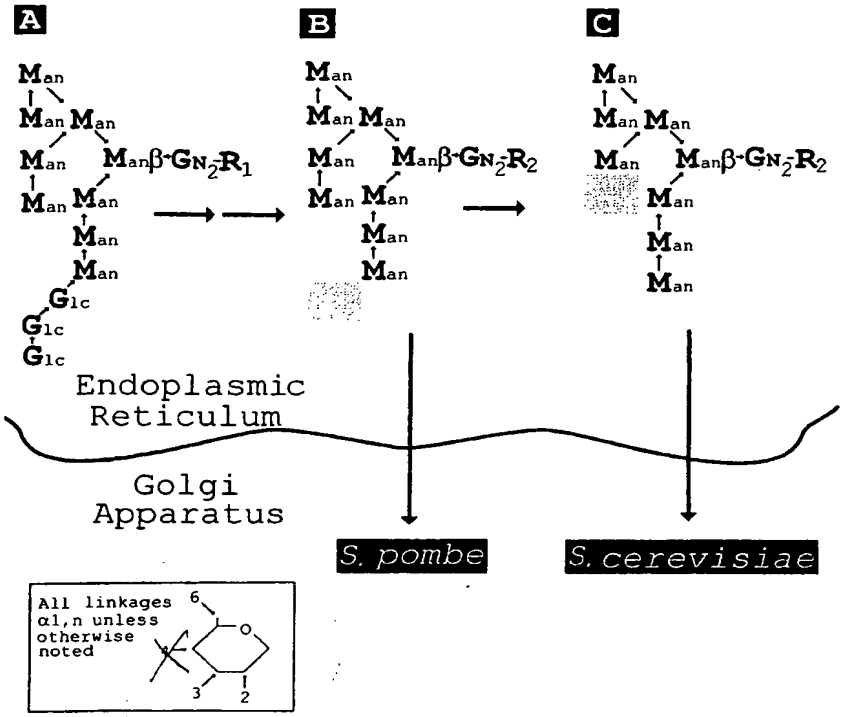


Fig. 1. Initial *N*-glycan processing in yeast. The nearly universal $Glc_3Man_9GlcNAc_2$ precursor (A), which is transferred from the lipid intermediate dolicholpyrophosphate (R_1) to protein (R_2), is trimmed to $Man_9GlcNAc_2$ (B) by processing glucosidases I and II. The $Man_9GlcNAc_2$ is then trimmed, except in *S. pombe*, to $Man_8GlcNAc_2$ by the ER Man_9-α-mannosidase (C).

synthesized as lipid-linked intermediates anchored to the ER membrane. The initial five mannoses are attached to chitobiose-PP-dolichol on the cytosolic face of the ER membrane using GDP-Man as a substrate. The remaining four mannoses and three glucoses are added on the luminal side of the ER using Dol-P-Man and Dol-P-Glc as the sugar donors, respectively. As discussed in Chapter 12, numerous asparagine linked glycosylation (*alg*) mutants have been isolated, many of which have provided insight to eukaryotic glycan processing pathways and their regulation through the cell cycle. The $Glc_3Man_9GlcNAc_2$ oligosaccharide is transferred cotranslationally en bloc to a nascent polypeptide chain by the oligosaccharyltransferase complex, which will be discussed in Chapter 4. Once transferred, the three glucoses are trimmed, and, with the exception of *Schizosaccharomyces pombe*, a specific mannose is removed from the $Man_9GlcNAc_2$ by the ER Man_9-α-mannosidase (Fig. 1), which will be detailed in the Chapter 5 on 'Processing glycosidases'. All eukaryotes examined to date, with the exception of *S. cerevisiae*, can transiently re-glucosylate malfolded glycoproteins in the ER, which is involved in the quality control of protein folding, a topic discussed further in Chapter 6. The glycoproteins are then transported to the Golgi apparatus, where oligosaccharide processing is quite variable between yeast species and vastly different than the Golgi processing events encountered in mammalian cells.

Yeasts do not trim farther than $Man_8GlcNAc_2$ as mammalian cells usually do, and therefore, with the exception of the nearly universal $Man_8GlcNAc_2$ and $Man_9GlcNAc_2$ *N*-glycan species, all yeast *N*-oligosaccharides contain a large high-mannose core, whose extensions are markedly different than those produced by mammalian cells. The genes for Golgi sugar transferases that have been identified and their products characterized to date will be discussed in Chapters 8 and 9. Yeasts do not add peripheral sialic acids to their glycans, a modification generally absent in single-celled eukaryotes; instead they use phos-

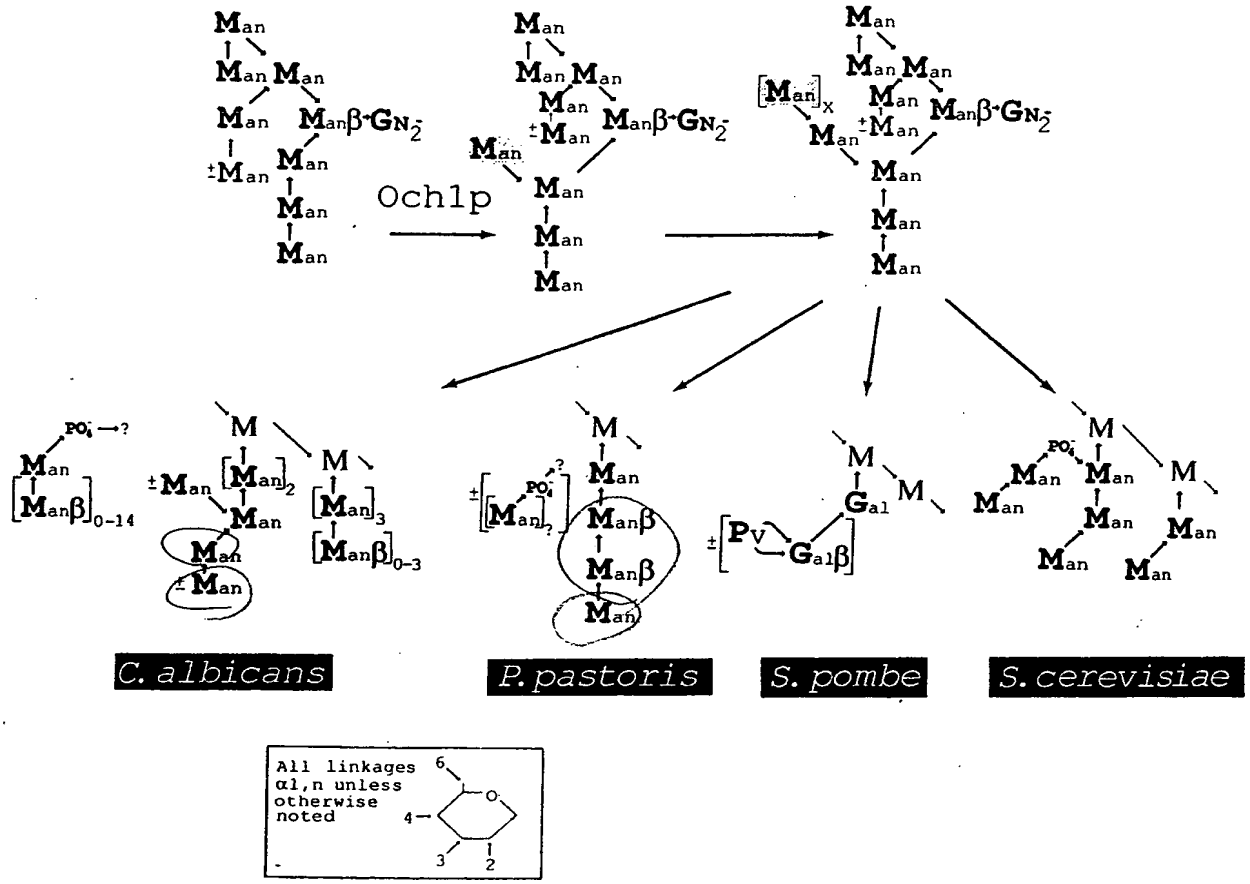


Fig. 2. *N*-Linked outer chain elongation in yeasts. The fully trimmed ER form of core *N*-glycan (Fig. 1B or C) is elongated by the addition of an α1,6-linked Man to its lower arm (top). Although the location of this addition is not known with certainty for many organisms, the similarities of this processing between fungi indicates that it is probably a conserved reaction. This Man is then extended by additional α1,6-linked Man residues. Side chains are attached to this backbone as shown on the bottom for each species as indicated. The linkage of the mannose phosphate side chain is not known for most organisms, and the structures shown for *Candida albicans* are a composite of those found in serotypes A and B. Pv stands for pyruvic acid (as a 4,6-attached *O*-[(*R*)-(1-carboxyethylidine) group).

phate, or in a few instances, glucuronic acid [16] or pyruvate [17] to impart negative charges to their *N*-glycans. The absolute functions of such charges in yeast glycoproteins remain unknown. The only higher eukaryotic parallel to the phosphate-containing yeast mannans is the addition of phosphate moieties to the high-mannose oligosaccharides of lysosomally targeted enzymes [18].

Yeasts generally add only mannose or mannose and galactose to their glycoprotein glycans. Some species add additional unusual monosaccharide units such as xylose [16,19]. Of the few yeasts suspected [20] of adding GlcNAc to their glycoprotein glycans, a common modification of mammalian oligosaccharides, only *Kluyveromyces lactis* is known to do so [21]. The antiporter for UDP-GlcNAc, required to supply GlcNAc for this addition in the *K. lactis* Golgi, has been recently cloned and characterized [22].

Yeasts elongate their oligosaccharides into two forms; a smaller ($Hex_{<15}$) 'core' series and larger ($Hex_{>15}$) 'outer chain' family of structures. The elongation process and some representative mannans are shown in Fig. 2. These elongated forms, often identified as mannan, phosphomannan, or galacto-

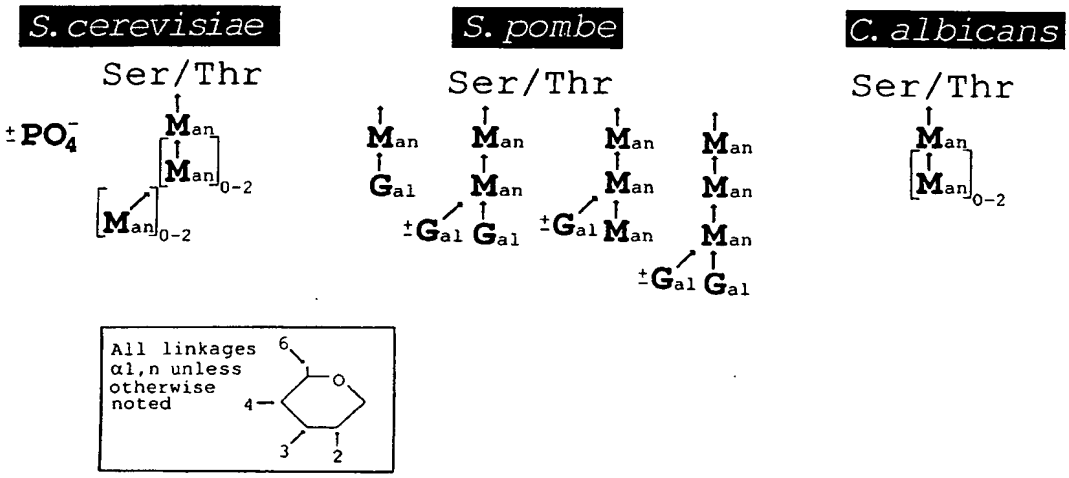


Fig. 3. Yeast *O*-glycan structures. *O*-Glycan structures from selected yeasts are identified by species. Note the $Man_{0-2}Man$-Ser/Thr core is common to all fungi studied to date.

mannan, have been at least roughly characterized from many yeasts. Although they differ among species in many respects, several features appear common among them all. The core-size oligosaccharides are elongated by addition of poly-α1,6-linked mannose 'backbone', which is minimally partially decorated with saccharide side-chains by substitution at the two-position. Yeasts, with a few exceptions, add diesterified phosphate to their elongated mannans, where the phosphate is linked by carbon-1 of a mannose to another mannose on the side-chains of the elongated oligosaccharide. The addition of these mannose phosphates is addressed below and in Chapter 10. Yeasts vary greatly in the linkage and composition of their mannan side chains; some are as short as a single Gal residue, while others are complex polymannose side-chains containing phosphate and sugar residues other than Man.

2.2. *O*-Glycosylation

O-Glycosylation is also vastly different in yeast (and in fungi in general) than in mammalian cells. Both organisms add monosaccharide residues directly to Ser and/or Thr. However, mammalian cells initiate synthesis by the addition of GalNAc residues in the Golgi apparatus using UDP-GalNAc as a sugar donor, while yeast and other fungi initiate *O*-linked synthesis by the addition of an initial mannose in the ER using Man-P-Dol as a sugar donor. Protein *O*-mannosylation in yeast will be further discussed in Chapter 7.

Mammalian cells elongate their *O*-GalNAc by the addition of Gal, GlcNAc, Fuc, and NeuNAc in later elements of the Golgi, and fungi elongate their *O*-Man by the addition of Man and/or Gal, also in the Golgi. Some representative yeast *O*-glycans are shown in Fig. 3. All yeasts studied to date can add Man to the *O*-linked Man to form Manα1,2Manα1,2Man-Ser/Thr, a structure which may [10] or may not [11] be antigenic in humans. Man_{1-3}-Ser/Thr structures are processed, depending upon the yeast species, by the addition of Gal or Man in various linkages to form structures $Hex_{<8}$ in length, which tend to be similar in nature to the side-chains found on their respective outer-chain mannans and core *N*-glycans. Unlike mammalian *O*-linked oligosaccharides, yeast *O*-glycans have rarely been reported to possess charged groups. The presence of mannose-phosphate on *O*-linked saccharides is discussed by Jigami and Odanis in Chapter 10.

3. Structural diversity

Other than those stated above, few generalizations can be made about yeast glycoprotein glycan struc-

tures. Some genera, such as *Schizosaccharomyces* [23] and *Saccharomyces* [24], *tend* to have similar structures (although *S. cerevisiae* and *S. kluverii* [25] are vastly different), while others, such as *Candida*, can have quite different structures not only between species, but between serotypes of the same species [16]. In addition, it has been shown that, at least in *S. cerevisiae*, the processing of the attached glycan can vary on different molecules of the same protein. Nevertheless, preferences have been observed for the type of oligosaccharide modification(s) found on specific sites of particular yeast glycoproteins [26,27].

Earlier works contain volumes of data on what are presumably yeast *N*- and *O*-linked glycans. Detailing these structures is beyond the scope of this overview, but they have been reviewed in some depth elsewhere [9,24,28–36]. Evaluation of these data requires caution, however, since often the oligosaccharide pools were prepared from cell-wall materials by harsh methods. The methods might have destroyed labile groups, or the starting materials may have contained adventitious oligosaccharide initially not covalently linked to the cell-wall glycoproteins. The sparse data on yeast *O*-glycans frequently reflects oligosaccharides prepared as 'alkali releasable' material, often without the protective agent sodium borohydride, which prevents the destruction of oligosaccharides by the 'peeling reaction' [37]. Some of these studies may have overlooked oligosaccharide species that were destroyed during β-elimination, or might include fragments of labile non *O*-linked oligosaccharide moieties, for example side chains from *N*-linked mannans or galactomannans.

Many yeast *N*-glycan structures were examined in the early literature for taxonomic purposes. Most of these data, however, should be viewed with caution, since the oligosaccharides were generally released from crude whole-cell fractions by harsh methods, and the analytical techniques employed were often not as definitive as those in current usage. Acetolysis has been one of the most utilized techniques in examining elongated fungal mannan structures. This procedure preferentially hydrolyzes the α1,6-Man bonds present in the mannan backbone, however α1,2- and α1,3-linkages are also susceptible to a lesser extent. In addition, any α1,6-linked side chains may also be destroyed [25]. Older versions of this procedure are known to degrade certain other labile linkages [38,39], and it is not known whether the milder procedures break any bonds other than the Manα1,6Man bond.

Some caveats also exist in examining products from exo- and endoglycosidase reactions; since every possible combination of linkages cannot be tested, it is unclear whether some linkage combinations expected to be glycosidase-sensitive may, in fact, be resistant. In addition, despite best efforts at purification, many sugar hydrolases still contain adventitious activities that could render product analysis inaccurate. Although the smaller glycoprotein glycans are usually more abundant on a per mole basis, the large, elongated *N*-oligosaccharides often make up the greatest percentage by weight of total carbohydrate and, in fact, were often the only structures determined in early studies. Finally, some of the data on yeast mannans may include data for mannose-containing chains not covalently attached to protein, such as those known to be present in certain species of *Cryptococcus* [19,35], *Pichia* [29,40], and *Hansenula* [29].

Oligosaccharide structural determination has been made more accurate and practical as a result of several analytical advances. The availability of cloned exo- and endoglycosidases, which allow the release of monosaccharides and *N*-glycans, has provided enzymatic tools of unparalleled purity and specificity. The endoglycosidases have allowed more quantitative discrimination between classes of *N*-glycans as well as between *N*-glycans and *O*-glycans. These mild enzymatic release methods allow the preparation of undamaged *N*-glycans for examination, in comparison to the potential structural effects resulting from chemical release. More sophisticated separation methods, such as high pH anion exchange chromatography (HPAEC), have allowed the separation of individual oligosaccharide isomers present in complex pools of structures. Refinements in high-field NMR spectroscopy have allowed unambiguous structural determinations. Compilations of currently used glycobiology techniques are available [41,42].

4. *N*- and *O*-glycans in specific yeast species

4.1. *S. cerevisiae*

S. cerevisiae is, by far, the most thoroughly studied of the yeasts. It is so commonly used that the unfortunate term 'yeast,' as opposed to 'a yeast,' is often employed when referring to *S. cerevisiae*, causing many to assume the described properties to be those of 'all yeasts'. The enzymes and processes involved in *S. cerevisiae* glycoprotein glycan processing have been reviewed elsewhere [7,43,44]. *S. cerevisiae*, like most fungi, does not trim the original $Glc_3Man_9GlcNAc_2$ added to nascent proteins past $Man_8GlcNAc_2$. Instead it directly elongates this structure into the 'core' or chain-elongated 'mannans'. Addition of an α1,2-linked man to the α1,6-linked Man added by Och1p [45,46] (Fig. 2) is the dedicated step determining whether the oligosaccharide is to remain a core size oligosaccharide or be elongated. The type of glycosylation, whether core or chain-elongated, is somewhat site-specific [26,47]. Certain glycosylation sites on each glycoprotein are preferentially elongated, while others tend to remain core-sized. This appears to be due, through steric factors, to the preferential impairment of glycosyltransferases by the tertiary structure of the folded substrate glycoprotein [26], a phenomenon occurring in higher eukaryotes as well [48]. Little information exists regarding outer-chain initiation or glycosylation-site specificity of other fungi. However, the structural similarities found between species seems to indicate that this process generally has been conserved.

Although evidence for a UDP-Gal antiporter exists in *S. cerevisiae* [49], no galactosylated species have ever been documented in this yeast. *S. cerevisiae* elongates its core *N*-glycans by the addition of α1,2-, α1,3- or α1,6-linked Man. The α1,2-additions are attached by the *Kre* and *Ktr* family of mannosyltransferases [50,51], and most α1,2-linked Man is decorated with α1,3-linked Man in a late Golgi compartment by Mnn1p [52]. As indicated earlier, the *ktr* and *mnn1* families of mannosyltransferases will be discussed in detail in Chapter 9. The structures and pathways of core glycosylation in *S. cerevisiae* have been determined [53]. In addition, as discussed in Chapter 10, mannose phosphoesters are attached to the 6-position of the branching Man of the elongated oligosaccharide chain, which are, in turn, elongated further. Although the attachment sites of these mannose phosphates to the neutral mannan has only been determined for a few species [54], it is assumed that most species attach this group as found in *S. cerevisiae* (Fig. 2). Two proteins, Mnn4p and Mnn6p, involved in the addition of the phosphoesters, have been examined [55–57].

S. cerevisiae forms *O*-linked glycans Man_{1-6} in length as shown in Fig. 3 [9]. The exact functions of these glycoprotein glycans in this organism is unknown at present, however, protein *O*-mannosylation appears required for survival [58]. The initial addition of Man to Ser/Thr occurs in the ER by the action of one of the *PMT* family of protein mannosyltransferases [58]. Although certain potential glycosylation sites become preferentially *O*-glycosylated, no obvious explanation exists for this preference [47]. Elongation of this *O*-mannose is then facilitated by the addition of mannoses by Mnn1p, and the *Kre* and *Ktr* family of mannosyltransferases in the compartments of the Golgi apparatus [50,51]. A second series of α1,3-mannosyltransferases that appear to be specific for substituting terminal α1,3-Man has recently been described [59]. Phosphate groups of unknown linkage are then attached to at least some of the *O*-glycans [47].

4.2. *Pichia pastoris*

P. pastoris produces core *N*-glycans of $Man_{8-11}GlcNAc_2$ in size [40] and also larger phosphomannans [38,60,61] as shown in Fig. 2. It is interesting to note that some glycoproteins expressed in this organism have the smaller *N*-glycans attached almost exclusively [40], while others have the large mannans attached almost exclusively [3]. The structures and pathways of core *N*-glycan synthesis have been determined and are known to commence with the addition of the archetypal α1,6-linked Man branch on the α1,3-linked core Man on the lower arm of the $Man_8GlcNAc_2$ precursor (Fig. 2) [40,62]. The elongated *N*-glycans are shorter than those from *S. cerevisiae* [62,63], and both the elongated and core oligosaccharides are known to contain phosphate [62,63], although the nature of the phosphate linkages presently is not known. Some members of the

Pichia and *Hansenula* families are known to produce large mannans, which are apparently not covalently attached to protein. They consist of mannose polymers of different linkage arrangements than the corresponding *N*-linked phosphomannans. Much of what is known about the closely related *Hansenula* species has been reviewed [7,34,35].

Little has been reported concerning the *O*-glycans of this organism, presumably due to the unusually small percentage of *O*-linked material in most glycoproteins tested to date [63]. Preliminary data using a recombinant protein highly *O*-glycosylated when expressed in this organism suggest that the *O*-glycans are similar to those of *S. cerevisiae* up to Man_3. More complicated, novel branched structures and some phosphorylated species also are produced (Trimble et al., Unpublished data).

4.3. S. pombe

S. pombe is rapidly gaining favor as a system for eukaryotic gene expression. Commercially available protein expression and purification kits are now sold and compilations of laboratory procedures specifically designed for this organism are available [64]. After removal of the three glucoses, *S. pombe* does not trim $Man_9GlcNAc_2$ to $Man_8GlcNAc_2$ in the ER [65,66] as seen in other organisms. It subsequently adds α1,2- and unusual α1,3-linked Gal to its core *N*-glycans [67]. Its elongated oligosaccharides (Fig. 2) are composed of a poly-α1,6-linked Man backbone decorated with α1,2-linked Gal [66,68,69]. Some of these 2-*O*-linked galactoses are modified by the addition of β1,3-linked Gal which, in turn, have attached 4,6-acetal-linked pyruvic acid. Reports of additional α1,2-linked Man on the elongated mannose and Galα1,2Gal being present in *S. pombe N*- and *O*-glycans [70] may be due to epitopes present under certain culture conditions or may be due to misidentified Galα1,2(Galα1,3)Man epitopes.

Several glycosylation-deficient mutants have been isolated in *S. pombe* [71,72]. Man-P-Dol synthase [73], a galactosyltransferase [71,74], the Gal epimerase [72], and the UDP-Gal transporter [75] have been cloned and/or purified. The Gal epimerase and Gal transporter mutants are viable and indicate that the Gal, and hence the pyruvylated Gal, are not essential, although some possible recognition function, as seen in the sea sponge *Aplysia kurodai* [76], cannot be discounted. In addition to the galactosyltransferases encoded by *gma12* and *gth1*, Dr. Thomas Chappell (personal communication) has found five ORFs with homology to *gth1*; *gth2–gth6*. The quad deletion *gma12*, *gth1*, *gth3*, *gth5* is nearly devoid of galactose as determined by flow cytometry quantitation with fluorescently labeled BSL I lectin.

S. pombe forms *O*-glycans [77] of $Gal_{0-2}Man_{1-3}$ in size as shown in Fig. 3. The inner mannose chain is the same as found in *S. cerevisiae*; Manα1,2Man and Manα1,2Manα1,2Man. One α1,2-linked Gal may be attached to their terminal Man by either gma12p or gth1p, and the α1,3-linked Gal are attached by a recently inferred branching enzyme(s), which appears to have specificity for α1,2-linked Man that are 2-*O*-substituted by either Gal or Man [77].

4.4. Candida albicans

C. albicans *N*-glycans have been extensively studied. The structures of its *N*-glycans, as well as those from additional *Candida* species and other pathogenic yeasts, have been reviewed [11,16,78]. The antigenic [16,79], lymphoproliferative [11], and immunosuppressive [78] effects of these glycans have also been reviewed. As described in these reviews, *C. albicans* produces varied mannan structures depending upon its serotype. Those shown in Fig. 2 detail a composite of structures from serotypes A and B. All pathogenic *Candida* species produce poly-β1,2-linked Man chains attached by phosphodiester linkages to their mannans. A β1,2-mannosyltransferase, which performs this synthesis, has been characterized from one species [80]. These β-linked chains specifically bind the organism to macrophages [81], and typically contain 2–6 Man residues but can contain up to 14 [82], depending on culture conditions. Although these phosphate-linked sidechains have been examined from numerous species in this family, the linkage of the phosphate to the main chain has yet to be determined in any species. *Candida* mannans also possess an epitope that binds interleukin 2 to a greater degree than the mannans from other yeast species [14]. Some *Candida* sp. *N*-glycan structures have been assigned subsequent to publication of the more recent reviews [80,83–89].

Although most *Candida* studies have focused on

the *N*-glycans, controversy exists regarding both the size and antigenicity of the *O*-glycans. *C. albicans* certainly produces *O*-glycans Hex_{1-3} in size [10,25,90,91], which are identical to those from *S. cerevisiae* as shown in Fig. 3 [10,25,91]. References to structural determinations preceding these reports appear to be elusive. Although some reports indicate the presence of additional Hex_{4-7} sized *O*-glycans [10,90], possibly containing β-linked mannoses, controversy exists whether these oligosaccharides represent *O*-glycans present at low levels or adventitious *N*-glycan fragments released from phosphoesters by the conditions used for β-elimination. The Hex_{1-3} size structures seem reasonable in light of those elucidated for other *Candida* species [83,85,92,93] *O*-glycans. The specificity of its PMT *O*-mannosylation has been examined [94], and it is known that *O*-glycans are formed initially from Man-P-Dol [95] and elongated with GDP-Man [96].

5. Concluding remarks

Although much is known about glycoprotein glycan processing in yeasts, there are many important gaps in our knowledge. Understanding yeast glycoprotein glycan processing is important for several reasons. Many of the processes are conserved between yeasts and other fungi, as well as between yeasts and mammals, even if the structures involved are not directly conserved. Determining these structures in pathogenic organisms may provide clues to targets for drug development, and may be important as diagnostic indicators of disease progression. Given the essential nature of the *O*-glycosidic linkage, what advantage might be given to fungi by adding Man to form *O*-glycans instead of the *N*-acetylhexosamine sugars added by all other studied eukaryotes, and can this difference be usefully exploited? Is there a common function to the acidic functionalities ubiquitously added to *N*-glycans, and can learning about these charged species in yeasts answer questions relevant to all eukaryotes? Filling the gaps in this knowledge will increase our understanding and abilities to exploit these commercially, biologically and medically relevant organisms, and will certainly give insight into the functioning of the glycoprotein glycans found in all eukaryotes.

Acknowledgements

We would like to thank Dr. Tom Chappell for information about the *S. pombe gth* family of *gma12* galactosyltransferase homologues prior to publication. We also are indebted to Dr. Shiego Suzuki, Dr. Nobuyuki Shibata and Dr. Robert D. Nelson for much helpful discussion and information regarding glycan structures found in *Candida* sp. We gratefully acknowledge the expert assistance of Ms. Tracy Godfrey in preparing the manuscript. Supported in part by USPH Grant GM23900 to R.B.T.

References

[1] M.K. Bennet, R.H. Scheller, Proc. Natl. Acad. Sci. USA 90 (1993) 2559–2563.
[2] S.W. Whitehart, E.W. Kubalek, Trends Cell Biol. 5 (1995) 64–68.
[3] W. Martinet, X. Saelens, T. Deroo, S. Nierynck, R. Contreras, W. Min Jou, W. Fiers, Eur. J. Biochem. 247 (1989) 332–338.
[4] P. Knight, Biotechnology 7 (1989) 35–40.
[5] J. Van Brunt, Biotechnology 4 (1986) 1057–1062.
[6] P. Yea, D. Landais, M. Lemaitre, I. Maury, J. Crenne, J. Becquart, A. Murry-Brelier, F. Boucher, G. Montay, R. Fleer, P. Hirel, J. Mayaux, D. Klatzmann, Proc. Natl. Acad. Sci. USA 89 (1992) 1904–1908.
[7] L. Lehle, W. Tanner, Protein glycosylation in yeast, in: J. Montruel, J.F.G. Vliegenthart, H. Schachter (Eds.), New Comprehensive Biochemistry, Vol. 29a: Glycoproteins, Elsevier, New York, 1995, pp. 475–509.
[8] D.T. Liu, Trends Biotechnol. 10 (1992) 114–120.
[9] C. Ballou, Adv. Microb. Physiol. 14 (1976) 93–155.
[10] M.P. Hayette, G. Strecker, C. Faille, D. Dive, D. Camus, D.W.R. MacKenzi, D. Poulain, J. Clin. Microbiol. 30 (1992) 411–417.
[11] R.P. Podzorski, G.R. Gray, R.D. Nelson, J. Immunol. 144 (1990) 707–716.
[12] T.R. Gemmill, R.B. Trimble, Glycobiology 8 (1998) 1087–1095.
[13] T. Kawai, Y. Suzuki, S. Eda, K. Ohtani, T. Kase, T. Sakamoto, H. Uemura, N. Wakamiya, Glycobiology 8 (1998) 237–244.
[14] J. Zanetta, R. Bonalay, S. Maschke, G. Strecker, J. Michalski, Glycobiology 8 (1998) 221–225.
[15] G. Cantos, C.L. Barbieri, M. Iacomini, P.A.J. Gorin, L.R. Travassos, Biochem. J. 289 (1993) 155–160.
[16] Y. Fukazawa, K. Kagaya, T. Shinoda, Curr. Top. Med. Mycol. 6 (1995) 189–219.
[17] T.R. Gemmill, R.B. Trimble, J. Biol. Chem. 42 (1996) 25945–25949.

[18] J.H. Stack, B. Horazdovski, S.D. Emr, Annu. Rev. Cell Dev. Biol. 11 (1995) 1–33.
[19] J.S. Schutzbach, H. Ankel, FEBS Lett. 5 (1969) 145–148.
[20] P.A.J. Gorin, J.F.T. Spencer, A.J. Finlayson, Carbohydr. Res. 16 (1970) 161–166.
[21] W.C. Raschke, C.E. Ballou, Biochemistry 11 (1972) 3807–3816.
[22] C. Abeijon, P.W. Robbins, C.B. Hirschberg, Proc. Natl. Acad. Sci. USA 93 (1992) 5963–5968.
[23] P.A.J. Gorin, J.F.T. Spencer, Can. J. Chem. 46 (1968) 2299–2304.
[24] C.E. Ballou, P.N. Lipke, W.C. Rashke, J. Bacteriol. 117 (1974) 461–467.
[25] N. Shibata, C. Kojima, Y. Satoh, A. Satoh, A. Suzuki, H. Kobayashi, S. Suzuki, Eur. J. Biochem. 217 (1993) 1–12.
[26] R.B. Trimble, F. Maley, F.K. Chu, J. Biol. Chem. 298 (1983) 2562–2567.
[27] F.D. Ziegler, F. Maley, R.B. Trimble, J. Biol. Chem. 263 (1988) 6986–6992.
[28] P.A.J. Gorin, J.F.T. Spencer, R.J. Magus, Can. J. Chem. 47 (1969) 3569–3576.
[29] E. Barreto-Bergter, P.A.J. Gorin, Adv. Carbohydr. Chem. Biochem. 41 (1983) 67–103.
[30] A.H. Rose, in: A.H. Rose (Ed.), The Yeasts, Vol. 2, 1st edn., Academic Press, New York, 1970, pp. 135–210.
[31] V. Farkas, in: A.H. Rose (Ed.), The Yeasts, Vol. 3, 2nd edn., Academic Press, New York, 1987, pp. 317–366.
[32] P.A.J. Gorin, J.F.T. Spencer, in: M.L. Wolfrom, R.S. Tipson (Eds.), Advances in Carbohydrate Chemistry and Biochemistry, Vol. 23, Academic Press, New York, 1968, pp. 367–417.
[33] P.A.J. Gorin, J.F.T. Spencer, Adv. Appl. Microbiol. 13 (1970) 25–91.
[34] C. Hashimoto, R.E. Cohen, C.E. Ballou, Biochemistry 19 (1980) 5932–5938.
[35] L. Lehle, in: W. Tanner, F.A. Loewus (Eds.), Encyclopedia of Plant Physiology, New Series, Vol. 13B: Plant Carbohydrates II, Springer-Verlag, Berlin, 1981, pp. 459–483.
[36] R.B. Trimble, F. Maley, W. Watorek, J. Biol. Chem. 256 (1981) 10037–10043.
[37] R.L. Whistler, J.N. BeMiller, Adv. Carbohydr. Chem. 13 (1958) 289–329.
[38] H. Kobayashi, N. Shibata, S. Suzuki, Arch. Biochem. Biophys. 245 (1986) 449–503.
[39] H. Kobayashi, N. Shibata, T. Yonezu, S. Suzuki, Arch. Biochem. Biophys. 256 (1987) 381–396.
[40] R.B. Trimble, P.H. Atkinson, J.F. Tschopp, R.R. Townsend, F. Maley, J. Biol. Chem. 34 (1991) 22807–22817.
[41] M. Fukuda, A. Kobata (Eds.), Glycobiology: A Practical Approach, Oxford University Press, New York, 1993.
[42] E.F. Hounsell (Ed.), Glycoprotein Analysis in Biomedicine, Humana Press, Totowa, NJ, 1993.
[43] P. Orlean, in: J.R. Pringle, J.R. Broach, E.W. Jones (Eds.), Molecular and Cell Biology of the Yeast Saccharomyces, Vol. 3, Cell Cycle and Cell Biology, Cold Spring Harbor Press, New York, 1997, pp. 229–362.
[44] R.B. Trimble, M.F. Verostek, Trends Genet. 7 (33) (1995) 1–30.
[45] T. Nagasu, Y. Shimma, Y. Nakanishi, J. Kuromitsu, K. Iwama, K. Nakayama, K. Suzuki, Y. Jigami, Yeast 8 (1992) 535–547.
[46] K. Nakayama, Y. Nakanishi-Shindo, A. Tanaka, Y. Haga-Toda, Y. Jigami, FEBS Lett. 412 (1997) 547–550.
[47] M.U. Jars, S. Osborn, J. Forstrom, V.L. MacKay, J. Biol. Chem. 270 (1995) 24810–24817.
[48] P. Hsieh, M.R. Rosner, P.W. Robbins, J. Biol. Chem. 258 (1983) 2548–2554.
[49] S.K. Roy, T. Yoko-o, H. Ikenaga, Y. Jigami, J. Biol. Chem. 273 (1998) 2583–2590.
[50] M. Lussier, A.M. Sdica, F. Bussereau, M. Jacquet, H. Bussey, J. Biol. Chem. 272 (1997) 15527–15531.
[51] M. Lussier, A.M. Sdicu, E. Winnett, D.H. Vo, J. Sheraton, A. Dusterhoft, R.K. Storms, H. Bussey, Yeast 13 (1997) 267–274.
[52] T.R. Graham, M. Seeger, G.S. Payne, V.L. MacKay, S.D. Emr, J. Cell Biol. 127 (1994) 667–678.
[53] R.B. Trimble, P.H. Atkinson, Glycobiology 2 (1992) 57–75.
[54] T.R. Theime, C.E. Ballou, Biochemistry 10 (1971) 4121–4129.
[55] T. Odani, Y. Shimma, A. Tanaka, Y. Jigami, Glycobiology 6 (1996) 805–810.
[56] E.M. Karson, C.E. Ballou, J. Biol. Chem. 253 (1978) 6484–6492.
[57] T. Odani, Y. Shimma, X. Wang, Y. Jigami, FEBS Lett. 420 (1997) 186–190.
[58] M. Gentzsch, W. Tanner, EMBO J. 15 (1996) 5752–5759.
[59] P.A. Romero, M. Lussier, S. Veronneau, A. Herscovics, H. Bussey, Glycobiology 7 (1997) 1045.
[60] P.A.J. Gorin, J.F.T. Spencer, S.S. Bhattacharjee, Can. J. Chem. 47 (1969) 1499–1505.
[61] P.A.J. Gorin, J.F.T. Spencer, Can. J. Microbiol. 18 (1972) 1709–1715.
[62] J.F. Tschopp, G. Sverlow, R. Kosson, W. Craig, L. Grinna, Biotechnology 5 (1987) 1305–1308.
[63] L.S. Grinna, J.F. Tschopp, Yeast 5 (1989) 107–115.
[64] C. Alfa, P. Fantes, J. Hyams, M. McLeod, E. Warbrick (Eds.), Experiments with Fission Yeast, Cold Spring Harbor Laboratory Press, New York, 1993.
[65] F.D. Ziegler, T.G. Gemmill, R.B. Trimble, J. Biol. Chem. 269 (1994) 12527–12535.
[66] C.E. Ballou, L. Ballou, G. Ball, Proc. Natl. Acad Sci. USA 91 (1994) 9327–9331.
[67] F.Z. Ziegler, J. Cavanagh, C. Lubowski, R.B. Trimble, Glycobiology 9 (1998) in press.
[68] P.A.J. Gorin, J.F.T. Spencer, Can. J. Chem. 46 (1968) 2299–2304.
[69] D.A. Bush, M. Horisberger, I. Horman, P. Wursch, J. Gen. Microbiol. 81 (1974) 199–206.
[70] L. Ballou, C. Ballou, Proc. Natl. Acad. Sci. USA 92 (1995) 2790–2794.
[71] T.G. Chappell, G. Warren, J. Cell Biol. 109 (1989) 2693–2707.

[72] K.M. Huang, M.D. Snider, Mol. Cell. Biol. 6 (1995) 485–496.
[73] J. Zou, J.R. Scocca, S.S. Krag, Arch. Biochem. Biophys. 317 (1995) 487–496.
[74] T.G. Chappell, M.A.N. Hajibagheri, K. Ayscough, M. Pierce, G. Warren, Mol. Cell. Biol. 5 (1994) 519–528.
[75] M. Tabuchi, N. Tanaka, S. Iwahara, K. Takegawa, Biochem. Biophys. Res. Commun. 232 (1997) 121–125.
[76] S. Araki, S. Abe, S. Ando, K. Kon, N. Fujiwara, M. Satake, J. Biol. Chem. 264 (1989) 19922–19927.
[77] T.R. Gemmill, R.B. Trimble, Glycobiology 9 (1998) in press.
[78] R.D. Nelson, N. Shibata, R.P. Podzorski, M.J. Herron, Clin. Microbiol. Rev. 4 (1991) 1–19.
[79] S. Suzuki, Curr. Top. Med. Mycol. 8 (1997) 57–70.
[80] A. Suzuki, N. Shibata, M. Suzuki, F. Saitoh, H. Oyamada, H. Kobayashi, S. Suzuki, Y. Okawa, J. Biol. Chem. 272 (1997) 16822–16828.
[81] R. Li, J.E. Cutler, J. Biol. Chem. 268 (1993) 18293–18299.
[82] P.A. Trinel, G. Lepage, T. Jouault, G. Strecker, D. Poulain, FEBS Lett. 416 (1997) 203–206.
[83] T. Hamada, T. Nakajima, K. Izaki, K. Matsuda, Eur. J. Biochem. 119 (1981) 365–371.
[84] N. Shibata, M. Onozawa, N. Tadano, Y. Hinosawa, A. Suzuki, K. Ikuta, H. Kobayashi, S. Suzuki, Y. Okawa, Arch. Biochem. Biophys. 336 (1996) 49–58.
[85] H. Kobayashi, M. Komido, M. Watanabe, K. Matsuda, M. Suzuki, T. Ikeda, H. Oyamada, N. Shibata, S. Suzuki, Infect. Immun. 62 (1994) 4425–4431.
[86] K. Ikuta, N. Shibata, H. Kanehiko, H. Kobayashi, S. Suzuki, Y. Okawa, FEBS Lett. 414 (1997) 338–342.
[87] N. Shibata, N. Senbongi, T. Hosoya, K. Kawahara, R. Akagi, A. Suzuki, H. Kobayashi, S. Suzuki, Y. Okawa, Eur. J. Biochem. 246 (1997) 477–485.
[88] H. Kobayashi, J. Suzuki, S. Tanaka, Y. Kiuchi, H. Oyamada, N. Iwadate, H. Suzuki, N. Shibata, S. Suzuki, Y. Okawa, Arch. Biochem. Biophys. 341 (1997) 70–74.
[89] N. Shibata, R. Akagi, T. Hosoya, K. Kawahara, A. Suzuki, K. Ikuta, H. Kobayashi, K. Hisamichi, Y. Okawa, S. Suzuki, J. Biol. Chem. 271 (1996) 9259–9266.
[90] Z. Kossaczka, J. Drgonova, B. Podobova, V. Betina, V. Farkas, Can. J. Microbiol. 41 (1995) 971–977.
[91] N. Shibata, T. Ichikawa, M. Tojo, M. Takahasi, N. Ito, Y. Okubo, S. Suzuki, Arch. Biochem. Biophys. 243 (1985) 338–348.
[92] H. Kobayashi, M. Takaku, Y. Nishidate, S. Takahashi, M. Takikawa, S. Shibata, S. Suzuki, Carbohydr. Res. 231 (1992) 105–106.
[93] H. Kobayashi, H. Mitobe, K. Takahashi, T. Yamamoto, N. Shibata, S. Suzuki, Arch. Biochem. Biophys. 294 (1992) 662–669.
[94] A. Weston, P.M. Nassau, C. Henly, M.S. Marriott, Eur. J. Biochem. 215 (1993) 845–849.
[95] B.L. Arroyo-flores, C. Calvo-Mendez, A. Flores-Carreon, E. Lopez-Romero, Microbiology 141 (1995) 2289–2294.
[96] M.S. Marriott, J. Gen. Microbiol. 103 (1977) 51–59.

APPENDIX II

PNAS
Proceedings of the National Academy of Sciences of the United States of America www.pnas.org

Use of combinatorial genetic libraries to humanize N-linked glycosylation in the yeast Pichia pastoris

Byung-Kwon Choi, Piotr Bobrowicz, Robert C. Davidson, Stephen R. Hamilton, David H. Kung, Huijuan Li, Robert G. Miele, Juergen H. Nett, Stefan Wildt, and Tillman U. Gerngross

PNAS 2003;100;5022-5027; originally published online Apr 17, 2003;
doi:10.1073/pnas.0931263100

Use of combinatorial genetic libraries to humanize N-linked glycosylation in the yeast *Pichia pastoris*

Byung-Kwon Choi*, Piotr Bobrowicz*, Robert C. Davidson*, Stephen R. Hamilton*, David H. Kung, Huijuan Li*, Robert G. Miele*, Juergen H. Nett*, Stefan Wildt*, and Tillman U. Gerngross†

Thayer School of Engineering, Dartmouth College, Hanover, NH 03755

Communicated by Phillips W. Robbins‡, Boston Medical Center, Boston, MA, March 4, 2003 (received for review December 5, 2002)

The secretory pathway of *Pichia pastoris* was genetically re-engineered to perform sequential glycosylation reactions that mimic early processing of N-glycans in humans and other higher mammals. After eliminating nonhuman glycosylation by deleting the initiating α-1,6-mannosyltransferase gene from *P. pastoris*, several combinatorial genetic libraries were constructed to localize active α-1,2-mannosidase and human β-1,2-*N*-acetylglucosaminyltransferase I (GnTI) in the secretory pathway. First, >32 N-terminal leader sequences of fungal type II membrane proteins were cloned to generate a leader library. Two additional libraries encoding catalytic domains of α-1,2-mannosidases and GnTI from mammals, insects, amphibians, worms, and fungi were cloned to generate catalytic domain libraries. In-frame fusions of the respective leader and catalytic domain libraries resulted in several hundred chimeric fusions of fungal targeting domains and catalytic domains. Although the majority of strains transformed with the mannosidase/leader library displayed only modest *in vivo* [i.e., low levels of mannose $(Man)_5$-$(GlcNAc)_2$] activity, we were able to isolate several yeast strains that produce almost homogenous N-glycans of the $(Man)_5$-$(GlcNAc)_2$ type. Transformation of these strains with a UDP-GlcNAc transporter and screening of a GnTI leader fusion library allowed for the isolation of strains that produce GlcNAc-$(Man)_5$-$(GlcNAc)_2$ in high yield. Recombinant expression of a human reporter protein in these engineered strains led to the formation of a glycoprotein with GlcNAc-$(Man)_5$-$(GlcNAc)_2$ as the primary N-glycan. Here we report a yeast able to synthesize hybrid glycans in high yield and open the door for engineering yeast to perform complex human-like glycosylation.

The number of protein-based therapeutics entering preclinical and clinical evaluation has shown robust growth and is expected to increase in the years to come. Fueled by advances in proteomics and genomics as well as the ability to engineer and humanize monoclonal antibodies, protein-based therapeutics constitute ≈500 candidates currently in clinical trials (1). Several therapeutic proteins can be made in a prokaryotic expression system such as *Escherichia coli* (e.g., insulin); however, the majority of therapeutic proteins require additional posttranslational modifications to attain full biological function. N-glycosylation in particular is essential for proper folding, pharmacokinetic stability, and efficacy for a large number of proteins (2). Most therapeutically relevant glycoproteins, including antibodies, are therefore expressed in mammalian cells. However, volumetric productivity, product heterogeneity, media cost, retroviral contamination, and the time required to generate stable cell lines are generally viewed as drawbacks of mammalian cell culture.

Fungal protein-expression systems do not suffer from the same limitations, and protein titers of 14.8 and 35 g/liter have been reported for secreted heterologous proteins in yeast and the filamentous fungus *Trichoderma reesei*, respectively (3, 4). However, glycoproteins derived from fungal expression systems contain nonhuman N-glycans of the high mannose (Man) type, which are immunogenic in humans and thus of limited therapeutic value (5).

Fungi and mammals share initial steps of protein N-glycosylation, which involves the site-specific transfer of $(Glc)_3$-$(Man)_9$-$(GlcNAc)_2$ from the luminal side of the endoplasmic reticulum (ER) to the *de novo* synthesized protein by an oligosaccharyltransferase complex. Subsequent trimming by glucosidases I and II and a specific ER-residing α-1,2-mannosidase leads to the formation of a $(Man)_8$-$(GlcNAc)_2$ structures (isomer Man8B) (Fig. 1), the N-glycan found on most glycoproteins leaving the ER.

After the export of predominantly $(Man)_8$-$(GlcNAc)_2$ containing glycoproteins to the Golgi, the pathways diverge notably between mammals and yeast (6). In the human Golgi α-1,2-mannosidases (IA–IC) remove Man to yield the $(Man)_5$-$(GlcNAc)_2$ structure, which forms the precursor for complex N-glycans (Fig. 1*A*). These mannosidases are typically type II membrane proteins with an N-terminal cytosolic tail, a transmembrane domain, a stem region, and a C-terminal catalytic domain (Fig. 1*B*). Localization of these proteins, as with most enzymes involved in Golgi glycosylation, is mediated by the cytosolic tail, the transmembrane region, and the stem (7).

In *Saccharomyces cerevisiae*, N-glycosylation has been studied extensively and, unlike mammalian N-glycan processing, involves the addition of numerous Man sugars throughout the entire Golgi, often leading to hypermannosylated N-glycan structures with >100 Man residues. This process is initiated in the early Golgi by an α-1,6-mannosyltransferase (Och1p) that prefers $(Man)_8$-$(GlcNAc)_2$ as a substrate but is able to recognize various other Man oligomers with the notable exception of the human $(Man)_5$-$(GlcNAc)_2$ intermediate, which is not a substrate (8). After addition of this first α-1,6-Man by Och1p, additional α-1,6-mannosyltransferases will extend the α-1,6 chain, which then becomes the substrate for medial- and trans-Golgi-residing α-1,2- and α-1,3-mannosyltransferases as well as phosphomannosyltransferases that add yet more Man sugars to the growing N-glycan structure (9). In *Pichia pastoris* a very similar process occurs; however, hypermannosylation occurs less frequently and to a lower extent. In addition α-1,3-mannosyltransferase activity has not been found in this yeast, and N-glycans from *P. pastoris* do not have α-1,3-Man attached to the outer Man chain (10).

Humanizing the glycosylation machinery of a yeast strain will require the (*i*) elimination of some endogenous glycosylation reactions and (*ii*) the recreation of the sequential nature of human glycosylation in the ER and Golgi. Although the first step involves the generation of gene knockouts (e.g., α-1,6- and/or α-1,3-mannosyltransferases), the second step requires the proper localization of active mannosidases, glycosyltransferases, and possibly nucleotide sugar transporters to specific organelles. Moreover the formation of certain sugar–nucleotide precursor pools such as CMP-sialic acid may have to be engineered into the yeast host. Much is known about the localization of endogenous proteins in the secretory pathway of *S. cerevisiae* and other yeasts; however, there is no reliable method to predict whether a Golgi protein from one

Abbreviations: Man, mannose; ER, endoplasmic reticulum; GnTI, β-1,2-*N*-acetylglucosaminyltransferase I; MALDI, matrix-assisted laser desorption ionization; K3, Kringle 3 domain of human plasminogen; TOF, time of flight.

*Present address: GlycoFi, Inc., 21 Lafayette Street, Suite 200, Lebanon, NH 03766.

†To whom correspondence should be addressed at: Thayer School of Engineering, Dartmouth College, 8000 Cummings Hall, Hanover, NH 03755. E-mail: tillman.gerngross@dartmouth.edu.

‡The member who communicated this paper is a Science Advisor for GlycoFi, Inc.

www.pnas.org/cgi/doi/10.1073/pnas.0931263100

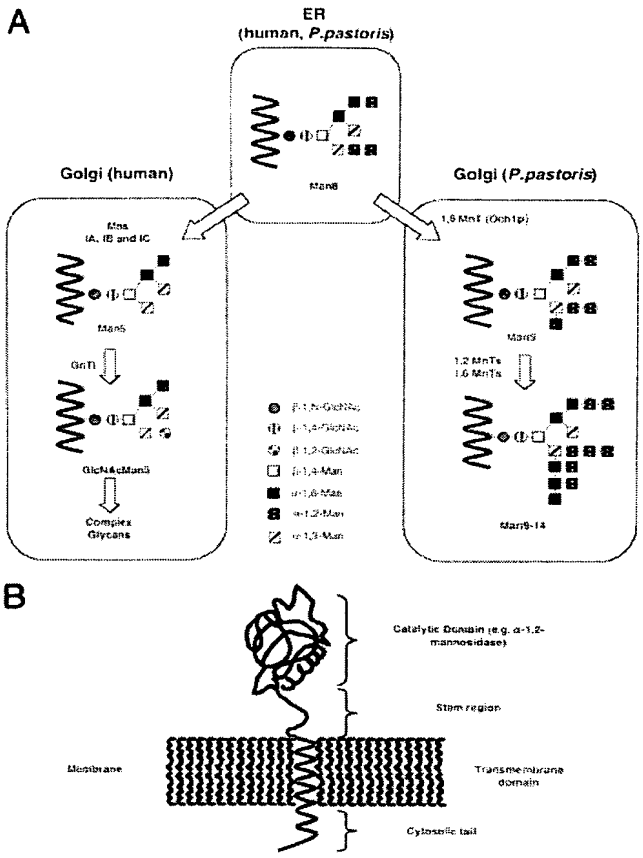


Fig. 1. (*A*) N-linked glycosylation pathway in humans and *P. pastoris*. Mns, α-1,2-mannosidase; MnT, mannosyltransferase; Och1p, the initiating 1,6-mannosyltransferase. (*B*) Structure of typical type II membrane glycosidase or glycosyltransferase.

organism will be properly localized in the Golgi of another organism. It is also unknown whether artificial in-frame protein fusions consisting of a yeast localization sequence and a catalytic domain from a nonyeast source (e.g., glycosyltransferases or mannosidases) will (*i*) localize to the desired organelle and (*ii*) show sufficiently high activity in the targeted environment. Although proper localization is important, it is not sufficient. Because the activity of the catalytic domain has to be maintained in the environment in which it has been localized, additional aspects such as pH optima must be considered. For example, Chiba *et al.* (11) found that localizing a mannosidase with a pH optimum of 5 in the ER of *S. cerevisiae* results in only modest intracellular mannosidase activity.

To overcome these major constraints we developed two tools: a combinatorial genetic library, generating hundreds of fusion constructs at a time, and a high-throughput screen that allows us to analyze large numbers of strains in parallel for their ability to modify N-glycans of recombinant reporter proteins. The combinatorial genetic library consists of an array of different fusion-protein constructs, each of which contains a fungal cellular targeting sequence fused in frame to a catalytic domain (e.g., mannosidase). Each of the 608 generated mannosidase fusion constructs was tested individually for its ability to catalyze the trimming of higher Man structures to $(Man)_5$-$(GlcNAc)_2$. Those strains that were able to generate mostly $(Man)_5$-$(GlcNAc)_2$ on a secreted reporter protein were subjected first to a second screen to ensure that trimming occurred *in vivo* and then engineered further to generate GlcNAc-$(Man)_5$-$(GlcNAc)_2$ by screening a similar β-1,2-*N*-acetylglucosaminyltransferase I (GnTI)/leader library. A more comprehensive article describing the characteristics of >600 leader/mannosidase and leader/GnTI fusions is in preparation (B.-K.C., P.B., R.C.D., S.R.H., A. Stadheim, H.L., R.G.M., J.H.N., S.W., and T.U.G., unpublished data); however, some of the most important findings are reported in this article.

Here we report the re-engineering of the secretory pathway in the methylotrophic yeast *P. pastoris*. The engineered strain produces predominantly N-glycans that are intermediates of the human glycosylation pathway, essentially void of fungal features. Our results suggest that further implementation of the described combinatorial library approach will allow for the engineering of yeast strains with increasingly human N-glycosylation. This article reports a genetically engineered yeast capable of producing a glycoprotein with a human-like hybrid N-glycosylation structure.

Materials and Methods

Strains, Culture Conditions, and Reagents. *E. coli* strains TOP10 or DH5α were used for recombinant DNA work. *P. pastoris* GS115 (*his4*, Invitrogen) or JC308 (*ura3*, *ade1*, *arg4*, *his4*, a gift from James M. Cregg, Keck Graduate Institute, Claremont, CA) were used for generation of yeast strains. Protein expression was carried out at room temperature in a 96-well-plate format with buffered glycerol-complex medium (BMGY) consisting of 1% yeast extract, 2% peptone, 100 mM potassium phosphate buffer (pH 6.0), 1.34% yeast nitrogen base, 4×10^{-5}% biotin, and 1% glycerol as a growth medium. The induction medium was buffered methanol-complex medium (BMMY) consisting of 1.5% methanol instead of glycerol in BMGY. Minimal medium is 1.4% yeast nitrogen base, 2% dextrose, 1.5% agar, and 4×10^{-5}% biotin and amino acids supplemented as appropriate. Restriction and modification enzymes were from New England BioLabs. Oligonucleotides were obtained from the Dartmouth College Core facility (Hanover, NH) or Integrated DNA Technologies (Coralville, IA). The enzymes, peptide *N*-glycosidase F, mannosidases, and oligosaccharides were obtained from Glyko (San Rafael, CA). Metal chelating HisBind resin was from Novagen. Lysate-clearing plates (96-well) were from Promega. Protein-binding 96-well plates were from Millipore. Salts and buffering agents were from Sigma. Matrix-assisted laser desorption ionization (MALDI) matrices were from Aldrich.

Cloning and Deletion of the *P. pastoris OCH1* Gene. The 1,215-bp ORF of the *P. pastoris OCH1* gene encoding a putative α-1,6-mannosyltransferase was amplified from *P. pastoris* genomic DNA (strain X-33, Invitrogen) by using the oligonucleotides 5′-ATGGCGAAGGCAGATGGCAGT-3′ and 5′-TTAGTC-CTTCCAACTTCCTTC-3′, which were designed based on the *P. pastoris OCHI* sequence (12). Subsequently, 2,685 bp upstream and 1,175 bp downstream of the ORF of the *OCHI* gene were amplified from a *P. pastoris* genomic DNA library (gift from Judah Folkman, Harvard Medical School, Boston) by using the internal oligonucleotides 5′-ATGGCGAAGGCAGATG-GCAGT-3′ and 5′-ACTGCCATCTGCCTTCGCCAT-3′ in the *OCHI* gene with T3 (5′-AATTAACCCTCACTAAAGGG-3′) and T7 (5′-GTAATACGACTCACTATAGGGC-3′) in the backbone of the library bearing plasmid λ ZAP II (Stratagene). The resulting 5,075-bp fragment was cloned into the pCR2.1-TOPO vector (Invitrogen) and designated pBK9. To create an *och1* knockout strain containing multiple auxotrophic markers, 100 μg of pJN329, a plasmid containing an *och1*::*URA3* mutant allele (J.F.N., S.W., and T.U.G., unpublished data; see Fig. 2*A* for details) was digested with *Sfi*I and used to transform *P. pastoris* strain JC308 by electroporation. After incubation on defined medium lacking uracil for 10 days at room temperature, 1,000 colonies were picked and restreaked. URA^+ clones that

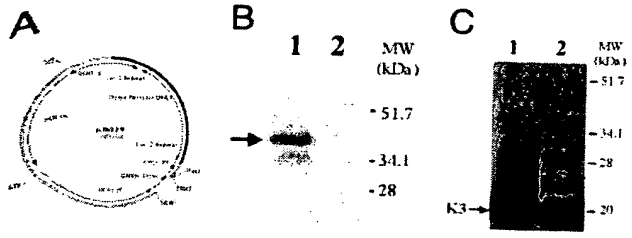


Fig. 2. Knockout of *OCH1* in *P. pastoris*. (*A*) The *OCH1* knockout plasmid pJN329 contains the *P. pastoris URA3* gene flanked by LacZ repeats and 2,878 bp upstream (5′) and 1,011 bp downstream (3′) of the *OCH1* gene of *P. pastoris*. (*B*) Immunoblot of Och1p in *Pichia* wild type (JC308, lane 1) and *och1* mutant (BK64-1, lane 2). The same amount of cell-free extract (50 μg per lane) was used, and Och1p was detected by using an Och1p peptide antibody followed by ECL. (*C*) The reporter protein K3 was expressed in *Pichia* wild type BK64 (lane 1) and an *och1* mutant, *P. pastoris* BK64-1 (lane 2). K3 was purified by Ni-affinity chromatography, separated by SDS/PAGE (4–20% gradient) under reducing conditions, and visualized by silver staining. The same amount of K3 (200 ng per lane) was loaded.

were unable to grow at 37°C but grew at room temperature were subjected to colony PCR to test for the correct integration of the *och1*::*URA3* mutant allele. One clone that exhibited the expected PCR pattern was designated YJN153.

Reporter Protein-Expression Construct. The Kringle 3 domain of human plasminogen (K3) was used as a model protein. A DNA fragment encoding the K3 (gift from Nick Menhart, Illinois Institute of Technology, Chicago) was amplified by using *Pfu* turbo polymerase (Stratagene) and cloned into *Eco*RI and *Xba*I sites of pPICZαA (Invitrogen), resulting in a C-terminal 6-His tag. To improve the N-linked glycosylation efficiency of K3 (13), Pro-46 was replaced with Ser-46 by using site-directed mutagenesis. The resulting plasmid was designated pBK64. The correct sequence of the PCR construct was confirmed by DNA sequencing.

Integration vectors and fusion constructs were generated based on the roll-in plasmids described by Lin Cereghino and coworkers (14); see details in *Supporting Methods*, which is published as supporting information on the PNAS web site, www.pnas.org.

Generation of Yeast Strains. To create *P. pastoris* strains expressing the K3, plasmid pBK64 was transformed into strains GS115 and YJN153, and colonies were selected on YPD medium containing 100 μg/ml zeocin to create strains BK64 and BK64-1, respectively. Plasmid pPB103 was linearized with *Eco*NI and transformed into strain BK64-1, and colonies were selected on defined medium lacking adenine. One strain containing the *Kluyveromyces lactis MNN2-2* gene was designated PBP1. Plasmids pBB27 and pBC4 were linearized with *Sal*I and transformed into strain PBP1, and colonies were selected on defined medium lacking histidine. Strains that were confirmed to contain the *Caenorhabditis elegans* α-1,2-mannosidase IB BB27 and BC4 fusion constructs were designated YJN188 and YJN168, respectively. Plasmid pNA15 was linearized with *Aat*II and transformed into strain YJN168, and colonies were selected on minimal medium without amino acids. One strain that was confirmed to contain the human GnTI gene fusion was designated YJN201.

Western Blotting. Proteins were separated by 4–20% gradient SDS/PAGE according to Laemmli (15) and then electroblotted onto nitrocellulose membrane (Schleicher & Schuell) as described (16). *P. pastoris* Och1p was detected by using an antibody raised against the peptide CQQLSSPKIDYDPLTL (Sigma–Genosys) with an ECL kit (Amersham Pharmacia).

Protein Purification. K3 was purified from the medium by Ni-affinity chromatography by using a 96-well format on a Beckman BioMek 2000 laboratory robot. The robotic purification is an adaptation of the protocol provided by Novagen for their HisBind resin.

Release of N-Linked Glycans. The glycans were released and separated from the glycoproteins by a modification of a previously reported method (17). After the proteins were reduced and carboxymethylated and the membranes were blocked, the wells were washed three time with water. The protein was deglycosylated by the addition of 30 μl of 10 mM NH_4HCO_3 (pH 8.3) containing 1 milliunit of *N*-glycanase (Glyko). After 16 h at 37°C, the solution containing the glycans was removed by centrifugation and evaporated to dryness.

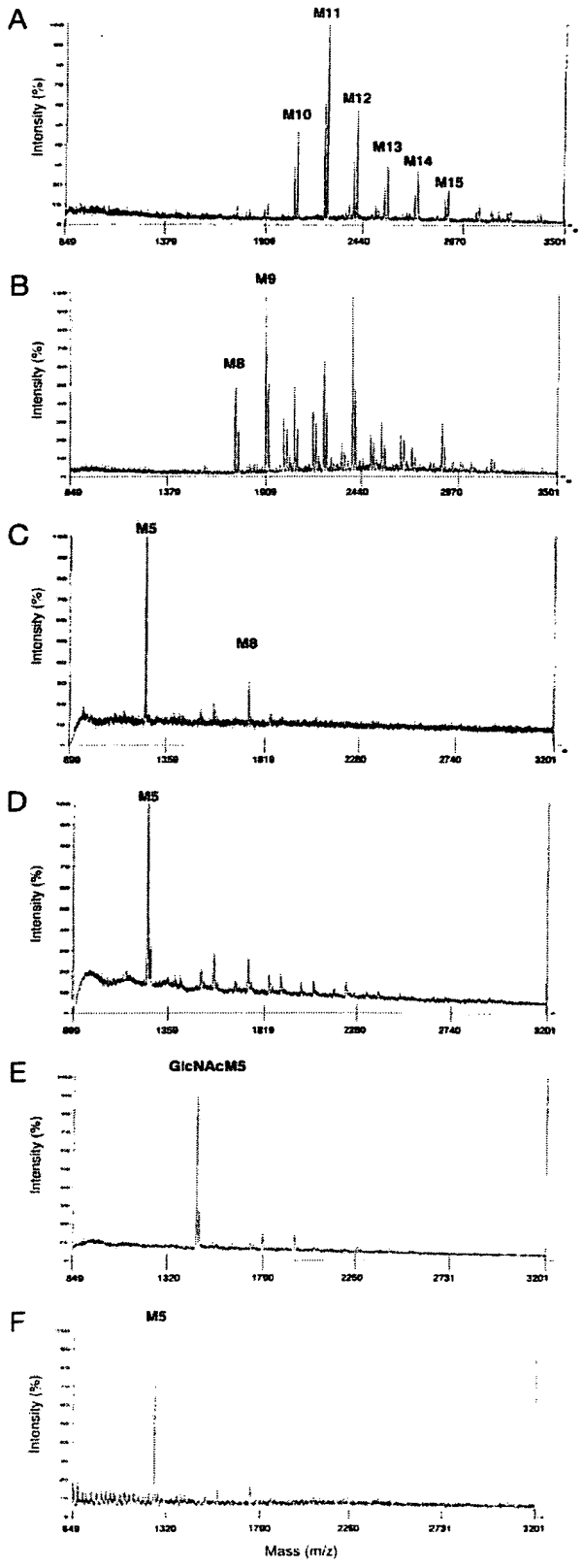
MALDI/Time-of-Flight (TOF) Mass Spectrometry. Molecular weights of the glycans were determined by using a Voyager DE PRO linear MALDI/TOF (Applied Biosciences) mass spectrometer with delayed extraction. The dried glycans from each well were dissolved in 15 μl of water, and 0.5 μl was spotted on stainless-steel sample plates and mixed with 0.5 μl of S-DHB matrix (9 mg/ml of dihydroxybenzoic acid/1 mg/ml of 5-methoxysalicylic acid in 1:1 water/acetonitrile/0.1% trifluoroacetic acid) and allowed to dry. Ions were generated by irradiation with a pulsed nitrogen laser (337 nm) with a 4-ns pulse time. The instrument was operated in the delayed extraction mode with a 125-ns delay and an accelerating voltage of 20 kV. The grid voltage was 93.00%, guide wire voltage was 0.1%, the internal pressure was $<5 \times 10^{-7}$ torr (1 torr = 133 Pa), and the low mass gate was 875 Da. Spectra were generated from the sum of 100–200 laser pulses and acquired with a 500-MHz digitizer. $(Man)_5$-$(GlcNAc)_2$ oligosaccharide was used as an external molecular weight standard. All spectra were generated with the instrument in the positive-ion mode.

Mannosidase Assays. Fluorescence-labeled $(Man)_8$-$(GlcNAc)_2$ (0.5 μg) was added to 20 μl of supernatant and incubated for 30 h at room temperature. After incubation the sample was analyzed by HPLC with an Econosil NH_2 4.6 × 250-mm, 5-μm bead, amino-bound silica column (Alltech, Avondale, PA). The flow rate was 1.0 ml/min for 40 min, and the column was maintained at 30°C. After eluting isocratically (68% A:32% B) for 3 min, a linear solvent gradient (68% A:32% B to 40% A:60% B) was used over 27 min to elute the glycans (18). Solvent A was acetonitrile, and solvent B was an aqueous solution of ammonium formate, 50 mM (pH 4.5). The column was equilibrated with solvent (68% A:32% B) for 20 min between runs.

Results

Generation of an α-1,6-Mannosyltransferase Deletion Mutant in *P. pastoris*. In *S. cerevisiae* the *OCH1* gene product (Och1p) is an α-1,6-mannosyltransferase that initiates the outer-chain elongation of N-linked glycans in the early Golgi. An *och1* null mutant strain in *S. cerevisiae* shows no α-1,6-Man linkage to the core glycan structure and consequently lacks hyperglycosylation (19). To generate a deletion of the *OCH1* homolog in *P. pastoris* an *och1*::*URA3* mutant allele was constructed and transformed into a *ura3* strain of *P. pastoris* (JC308). Several *och1* mutant strains were identified and confirmed by PCR and Western blotting (Fig. 2*B*). The *P. pastoris och1* mutant strain exhibited temperature sensitivity and increased flocculation similar to that observed in *S. cerevisiae* (data not shown).

Glycan structures of heterologous proteins expressed in *P. pastoris* are heterogeneous, mostly consisting of $(Man)_{10-16}$-$(GlcNAc)_2$ mannans with varying degrees of charged glycans (10, 20). To monitor the effect of different engineering steps on the glycosylation *of P. pastoris*, a secreted form of K3 was used as a reporter protein. Hyperglycosylation observed by SDS/PAGE



analysis when K3 was expressed in a wild-type *P. pastoris* strain was eliminated in the *och1* mutant strain (Fig. 2*C*). N-glycans released from secreted K3 were analyzed directly by MALDI/TOF mass spectrometry. N-glycans of K3 expressed in a *P. pastoris* wild-type strain were found to have a molecular mass consistent with $(Man)_{10-16}$-$(GlcNAc)_2$, confirming previous findings in this yeast (Fig. 3*A*). By comparison, the N-glycans of K3 in an *och1* mutant strain revealed predominantly $(Man)_{8-12}$-$(GlcNAc)_2$, representing a noticeable shift to smaller glycans (Fig. 3*B*) and a complete elimination of the smearing observed in the SDS/PAGE analysis (Fig. 2*C*). To rule out the possibility of protein-specific interactions a second reporter protein, full-length human IFN-β, was used and revealed identical results (data not shown).

Construction of ER/Golgi leader, α-1,2-Mannosidase, and GnTI Libraries. To sequentially localize different mannosidases and GnTIs along the early secretory pathway of *P. pastoris* three separate gene libraries were designed. The first library (the leader library) contained DNA fragments encoding N-terminal peptides of known type II membrane proteins that either localize in the ER or Golgi of *S. cerevisiae* and *P. pastoris*. They include Gls1, Mns1, Sec12, Mnn9, Van1, Anp1, Hoc1, and Mnn10, Mnn11 from *S. cerevisiae* and Och1 and Sec12 from *P. pastoris*. The generation of the respective DNA constructs is exemplified by the construction of Mns1, Mnn9, and Mnn10 leaders in *Materials and Methods*. A second library contained catalytic domains of α-1,2-mannosidases from *Homo sapiens*, *Mus musculus*, *Aspergillus nidulans*, *C. elegans*, *Drosophila melanogaster*, and *Penicillium citrinium*. The generation of the respective DNA constructs is exemplified by the construction of mannosidase IB from *C. elegans* in *Materials and Methods*. Finally, a third library contained catalytic domains of GnTI genes from *H. sapiens*, *C. elegans*, *Xenopus laevis*, *and D. melanogaster*. All libraries were designed in a way that any combination of a leader construct and a catalytic domain created a gene encoding a chimeric fusion protein.

Each leader fragment was represented in three lengths. The short form encoded the N-terminal cytoplasmic tail and the transmembrane domain. The long form encoded additional residues containing the complete stem region up to the respective catalytic domain, which was determined by sequence homology to known catalytically active fragments of such enzymes. The medium form was an intermediate version containing approximately half of the stem region in addition to the sequence encoded in the short form. Catalytic domains were selected to cover a wide range of pH optima as determined from literature data (e.g., *P. citrinium* and *M. musculus*) and temperature optima by selecting domains from organisms that exist at different temperatures (e.g., *C. elegans* and *H. sapiens*). Each catalytic domain was represented in several lengths and generally lacked the native N-terminal cytosolic and transmembrane domain. Some of the catalytic domains were selected solely on the basis

Fig. 3. Positive-ion MALDI/TOF mass spectra of N-linked glycans released from K3. K3 was produced in *P. pastoris* strains BK64, BK64-1, YJN168, YJN188, and YJN201 and purified from culture supernatants by Ni-affinity chromatography. The glycans were released from K3 by peptide *N*-glycosidase F treatment. The released N-linked glycans were analyzed by MALDI/TOF mass spectrometry, typically appearing as the sodium or potassium adducts. (*A*) BK61, wild-type strain of *P. pastoris* expressing K3. (*B*) BK64-1, *och1* deletion expressing K3. (*C*) YJN168, *och1* deletion expressing K3, *K. lactis* UDP-GlcNAc transporter, and *C. elegans* α-1,2-mannosidase IB fused to *MNS1*. (*D*) YJN188, *och1* deletion expressing K3, *K. lactis* UDP-GlcNAc transporter, and *C. elegans* α-1,2-mannosidase IB fused to *MNN10*. (*E*) YJN201, *och1* deletion expressing K3, *K. lactis* UDP-GlcNAc transporter, *C. elegans* α-1,2-mannosidase IB fused to *MNS1*, and human GnTI fused to *MNN9*. (*F*) YJN201 after β-*N*-acetylhexosaminidase treatment. M, Man.

Table 1. Relative amount of $(Man)_5$ on secreted K3

Amount of $(Man)_5$ on secreted K3, % of total glycans	Number of constructs (%)
ND*	19 (3.1)
0–10	341 (56.1)
10–20	50 (8.2)
20–40	75 (12.3)
40–60	72 (11.8)
>60	51 (8.4)†
Total	608 (100)

Six hundred and eight different strains of *P. pastoris* (*och1*) were generated by transforming them with a single construct of a combinatorial genetic library that was generated by fusing 19 α-1,2-mannosidase catalytic domains to 32 fungal ER and cis-Golgi leaders.

*Several fusion constructs were not tested because the corresponding plasmids could not be propagated in *E. coli* before transformation into *P. pastoris*.

†Clones with the highest degree of $(Man)_5$ trimming (30/51) were analyzed further for mannosidase activity in the supernatant of the medium. The majority (28/30) displayed detectable mannosidase activity in the supernatant (e.g. Fig. 4*B*). Only two constructs displayed high $(Man)_5$ levels while lacking mannosidase activity in the medium (e.g. Fig. 4*C*).

of sequence homology to other known α-1,2-mannosidases (e.g. *C. elegans*) or GnTIs and had not been characterized previously.

After screening the fusion libraries chimeric constructs were identified that displayed a high degree of Man-trimming activity and UDP-GlcNAc transfer activity on the reporter protein K3. A detailed analysis of the characteristics of the fusion libraries will be published elsewhere (B.-K.C., P.B., R.C.D., S.R.H., A. Stadheim, H.L., R.G.M., J.H.N., S.W., and T.U.G., unpublished data).

Expression of α-1,2-Mannosidase Fusion Constructs in a *P. pastoris och1* Mutant Strain. After screening a library of 608 leader/α-1,2-mannosidase fusions targeted to the ER and early Golgi, several clones were identified that produced N-glycans consistent with a mass of $(Man)_5$-$(GlcNAc)_2$. Specifically, a putative *C. elegans* homolog of known α-1,2-mannosidases showed a high degree of trimming to $(Man)_5$-$(GlcNAc)_2$ when fused to both the *S. cerevisiae MNS1* and *MNN10* leader-encoding fragments. Although these constructs and several others resulted in glycans, which were 70–80% or more $(Man)_5$-$(GlcNAc)_2$ (Fig. 3 *C* and *D*), >56% of the fusions resulted in <10% $(Man)_5$-$(GlcNAc)_2$ (Table 1). These data clearly emphasize the importance of choosing the proper combination of (*i*) a localization sequence and (*ii*) an α-1,2-mannosidase catalytic domain of the proper length. Fungal α-1,2-mannosidases with acidic pH optima (e.g., *P. citrinium* and *A. nidulans*), when expressed as fusions with the leader library, generally resulted in low $(Man)_5$-$(GlcNAc)_2$ yields (data not shown) consistent with previous findings (11, 21).

Because previous researchers have found that trimming to $(Man)_5$-$(GlcNAc)_2$ was often accompanied by leakage of mannosidase into the medium (22), we further investigated whether Man trimming occurred *in vivo*, in the Golgi, or *ex vivo* after secretion of the protein into the medium. To determine the extent of mannosidase activity in the medium, 2-aminobenzamide-labeled $(Man)_8$-$(GlcNAc)_2$ was used to assay the culture supernatant. Many of the efficient $(Man)_5$-$(GlcNAc)_2$-producing constructs displayed a high degree of mannosidase activity in the supernatant, suggesting that at least some of the observed $(Man)_5$-$(GlcNAc)_2$ structures were produced *ex vivo*. However, by applying a double screen we were able to identify specific chimeric fusions that were entirely retained intracellularly while at the same time displaying high *in vivo* α-1,2-mannosidase activity (Fig. 4*C*).

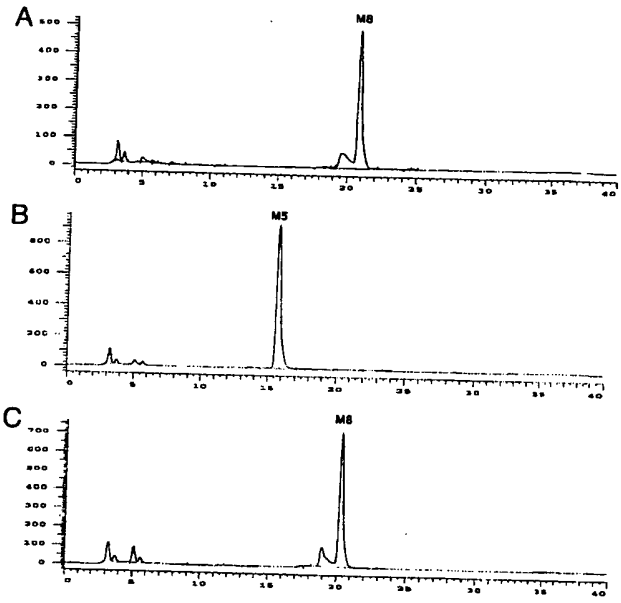


Fig. 4. Extracellular activity of *C. elegans* α-1,2-mannosidase IB. 2-Aminobenzamide-labeled $(Man)_8$-$(GlcNAc)_2$ was incubated with culture supernatants at 25°C for 30 h and analyzed by HPLC. (*A*) $(Man)_8$-$(GlcNAc)_2$ standard in BMMY medium (no cells). (*B*) Supernatant from YJN188. (*C*) Supernatant from YJN168. M, Man.

Expression of GnTI Fusion Constructs in a *P. pastoris och1* Mutant Strain Containing an Active α-1,2-Mannosidase. Further humanization of $(Man)_5$-$(GlcNAc)_2$ glycan structures involves the subsequent *in vivo* conversion of $(Man)_5$-$(GlcNAc)_2$ to GlcNAc-$(Man)_5$-$(GlcNAc)_2$. This step requires UDP-GlcNAc as a substrate and involves the transfer of GlcNAc to $(Man)_5$-$(GlcNAc)_2$ by GnTI (Fig. 1). To ensure sufficient levels of UDP-GlcNAc in the Golgi we cloned the UDP-GlcNAc transporter from *K. lactis* into an *och1* mutant strain, which also displays high *in vivo* mannosidase activity.

A leader/GnTI fusion library containing 67 constructs was screened. Among several active fusion constructs, one consisting of human GnTI and a leader sequence from *S. cerevisiae MNN9* was particularly active, and the corresponding strains yielded K3-containing glycans with a mass consistent with GlcNAc-$(Man)_5$-$(GlcNAc)_2$ almost exclusively (Fig. 3*E*). Moreover, these glycans were converted completely to $(Man)_5$-$(GlcNAc)_2$ by *in vitro* *N*-acetylhexosaminidase digestion, further indicating that this strain secretes protein almost uniformly modified with glycans of the structure GlcNAc-$(Man)_5$-$(GlcNAc)_2$, a well described human glycosylation intermediate (Fig. 3*F*). Importantly, GnTI activity was assayed in the culture medium of this strain and found to be absent (data not shown). Finally, to determine whether the UDP-GlcNAc transporter was required to provide sufficient amounts of substrate for GnTI, a strain producing $(Man)_5$-$(GlcNAc)_2$ was transformed with the same GnTI construct in the absence of the transporter. Although some GlcNAc-$(Man)_5$-$(GlcNAc)_2$ was observed from the purified K3, a large percentage of glycans consistent with $(Man)_5$-$(GlcNAc)_2$ remained, indicating that GlcNAc transfer was less efficient than in the strain containing the transporter (data not shown). This result confirms the purported necessity of a UDP-GlcNAc transporter for efficient GlcNAc transfer originally found in *K. lactis* (23).

Discussion

P. pastoris is one of several yeasts capable of high-level production of heterologous glycoproteins (24). As with any other fungal

protein-expression systems, the heterologous glycoprotein is glycosylated in a fungal-like fashion generally involving the addition of α-1,6- and α-1,2-linked Man and mannosylphosphate to the $(Man)_8$-$(GlcNAc)_2$ core (Fig. 1; ref. 25). This yeast-type glycosylation pattern is recognized by the human immune system, which renders the underlying protein unfit for therapeutic use. In view of the current shortage of efficient glycoprotein-expression systems we have focused our efforts on engineering commercially relevant yeast and filamentous fungi to produce N-glycans with human-like glycosylation structures. In this study we report the construction of a strain that produces high levels of a reporter protein modified with a human glycosylation intermediate in the *P. pastoris* system.

The first step in attaining complex human-type glycans in a fungal system is to eliminate yeast-type glycosylation. To accomplish this, the *P. pastoris OCH1* gene was deleted in a strain secreting the reporter protein K3. Yeast-type hyperglycosylation was abrogated in this strain as observed by silver staining of secreted K3 (Fig. 2*C*), and when released N-glycans were analyzed by MALDI/TOF, a general trend toward smaller structures was observed (Fig. 3*B*).

Once an appropriate core high-Man glycan is obtained, the next immediate step in the conversion to human-type N-glycans involves the functional expression and localization of an α-1,2-mannosidase. This enzyme will trim the $(Man)_8$-$(GlcNAc)_2$ core structure to $(Man)_5$-$(GlcNAc)_2$ and thereby generate the structure that is capable of receiving the GlcNAc that initiates the formation of hybrid N-glycans. A very similar approach was taken in a triple mutant *och1 mnn1 mnn4* strain of *S. cerevisiae* (11). ER localization of a fungal α-1,2-mannosidase (from *Aspergillus saitoi*) was accomplished by adding the tetrapeptide HDEL as an ER retrieval tag to the C terminus of the gene. By using the *S. cerevisiae* GAPDH promoter and a multicopy number plasmid, mannosidase activity was detectable in cell-free extracts; however, only 27% of the N-glycans of an endogenous marker protein (carboxypeptidase Y) were trimmed from $(Man)_8$-$(GlcNAc)_2$ to $(Man)_5$-$(GlcNAc)_2$ *in vivo*. Although not entirely successful, the pioneering work of Chiba *et al.* (11) demonstrates that N-glycans from *S. cerevisiae* can be modified substantially by engineering glycosylation pathways. Here the localization of catalytic domains to the secretory pathway of the related yeast *P. pastoris* using yeast type II membrane protein leader domains is demonstrated. The benefit of a large library of leader domains along with an equally diverse library of catalytic domains allowed for the selection of the most active fusion constructs from a pool of >600 candidates, many of which were marginally active or not at all. Despite the lack of success of many constructs, a few particularly active enzyme fusions were able to trim the core $(Man)_{8-12}$-$(GlcNAc)_2$ glycans observed in the *och1* mutant strain to $(Man)_5$-$(GlcNAc)_2$. Two of these constructs described here, which are fusions of a putative *C. elegans* α-1,2-mannosidase with different yeast type II leader domains, are able to do so at high efficiency (>75%).

The correct localization of enzymes involved in glycosylation is critical to allow for the sequential glycan modifications as glycoproteins proceed through the secretory pathway. However, one additional concern has come from another previous attempt to engineer glycans in *P. pastoris*, which was undertaken with an α-1,2-mannosidase from *T. reesei* (22). In this study immunofluorescent microscopy was used to demonstrate that a mannosidase-myc-HDEL fusion localized primarily in the ER of *P. pastoris*; however, leakage into the medium was also observed by Western blotting. Because the secreted glycoprotein of interest will be in the supernatant for many hours, the cosecretion of mannosidases into the medium is of concern because it can be misinterpreted as *in vivo* activity. It is important to generate $(Man)_5$-$(GlcNAc)_2$ structures *in vivo*, and early in the secretory pathway, if subsequent conversion to complex glycans is to be achieved. Although it is well established that the *ERD2*-based retrieval system is leaky and retention of the HDEL-tagged mannosidase in the secretory pathway cannot be ensured (26), the same concern is emphasized further by the striking difference demonstrated here between the two different *C. elegans* α-1,2-mannosidase IB fusion constructs. Thus, the use of a series of different fusion constructs with many differentially localized type II domains has allowed us to screen for chimera with activity that is completely *in vivo*.

After efficient trimming of the core glycan to $(Man)_5$-$(GlcNAc)_2$ the next step in the conversion of high Man-type glycans to hybrid- and complex-type glycans involves the expression and localization of the enzyme GnTI. Here again a library of GnTI catalytic domains was used and allowed for the screening for and selection of several particularly active GnTI fusion constructs for further study. One particularly active fusion using the human GnTI catalytic domain shown here converts the $(Man)_5$-$(GlcNAc)_2$ substrate to the desired GlcNAc-$(Man)_5$-$(GlcNAc)_2$ product almost quantitatively, an activity that was shown to be completely *in vivo*. Furthermore, the demonstrated activity of GnTI on the $(Man)_5$-$(GlcNAc)_2$ substrate is the best evidence that the α-1,2-mannosidase activity is indeed occurring in the secretory pathway. This is a demonstration of a high-level hybrid N-glycan modification of a secreted protein in yeast and represents a significant step toward the ability to express fully human glycoproteins in yeast. Although the generated structures are expected to be nonimmunogenic in humans, additional Man removal (i.e., the removal of 1,6- and 1,3-Man from the trimannose core) and further addition of β-1,2-GlcNAc will be required to generate complex N-glycans of therapeutic utility (e.g., for the production of monoclonal antibodies).

We thank Beata Bobrowicz, Teresa Mitchell, Sebastian Rausch, Andy Stadheim, Amelia Walling, and Harry Wischnewski for technical assistance. We gratefully acknowledge the gift of strains and reagents from Judah Folkman, James Cregg, Nick Menhart, Robert Barsteadt, Carlos Hirschberg, and Takashi Yoshida. We also thank Phil Robbins and Roger Bretthauer for their encouragement and continued support.

1. Walsh, G. (2000) *Nat. Biotechnol.* **18,** 831–833.
2. Helenius, A. & Aebi, M. (2001) *Science* **291,** 2364–2369.
3. Werten, M. W. T., van den Bosch, T. J., Wind, R. D., Mooibroek, H. & de Wolf, F. A. (1999) *Yeast* **15,** 1087–1096.
4. Durand, H. & Clanet, M. (1988) *Enzyme Microb. Technol.* **10,** 341–346.
5. Ballou, C. E. (1990) *Methods Enzymol.* **185,** 440–470.
6. Hubbard, S. C. & Ivatt, R. J. (1981) *Annu. Rev. Biochem.* **50,** 555–583.
7. Gleeson, P. A. (1998) *Histochem. Cell Biol.* **109,** 517–532.
8. Nakayama, K., Nakanishi-Shindo, Y., Tanaka, A., Haga-Toda, Y. & Jigami, Y. (1997) *FEBS Lett.* **412,** 547–550.
9. Dean, N. (1999) *Biochim. Biophys. Acta* **1426,** 309–322.
10. Bretthauer, R. K. & Castellino, F. J. (1999) *Biotechnol. Appl. Biochem.* **30,** 193–200.
11. Chiba, Y., Suzuki, M., Yoshida, S., Yoshida, A., Ikenaga, H., Takeuchi, M., Jigami, Y. & Ichishima, E. (1998) *J. Biol. Chem.* **273,** 26298–26304.
12. Koji, M., Narutoshi, S. & Tomoyasu, R. (1996) Jpn. Patent JP 8336387.
13. Hayes, M. L., Bretthauer, R. K. & Castellino, F. J. (1975) *Arch. Biochem. Biophys.* **171,** 651–655.
14. Lin Cereghino, G. P., Lin Cereghino, J., Sunga, A. J., Johnson, M. A., Lim, M., Gleeson, M. A. & Cregg, J. M. (2001) *Gene* **263,** 159–169.
15. Laemmli, U. K. (1970) *Nature* **227,** 680–685.
16. Towbin, H. & Gordon, J. (1984) *J. Immunol. Methods* **72,** 313–340.
17. Papac, D. I., Briggs, J. B., Chin, E. T. & Jones, A. J. S. (1998) *Glycobiology* **8,** 445–454.
18. Turco, S. J. (1981) *Anal. Biochem.* **118,** 278–283.
19. Nakanishi-Shindo, Y., Nakayama, K. I., Tanaka, A., Toda, Y. & Jigami, Y. (1993) *J. Biol. Chem.* **268,** 26338–26345.
20. Trimble, R. B., Atkinson, P. H., Tschopp, J. F., Townsend, R. R. & Maley, F. (1991) *J. Biol. Chem.* **266,** 22807–22817.
21. Martinet, W., Maras, M., Saelens, X., Jou, W. M. & Contreras, R. (1998) *Biotechnol. Lett.* **20,** 1171–1177.
22. Callewaert, N., Laroy, W., Cadirgi, H., Geysens, S., Saelens, X., Jou, W. M. & Contreras, R. (2001) *FEBS Lett.* **503,** 173–178.
23. Guillen, E., Abeijon, C. & Hirschiberg, C. B. (1998) *Proc. Natl. Acad. Sci. USA* **95,** 7888–7892.
24. Lin Cereghino, J. & Cregg, J. M. (2000) *FEMS Microbiol. Rev.* **24,** 45–66.
25. Miele, R. G., Castellino, F. J. & Bretthauer, R. K. (1998) *Biotechnol. Appl. Biochem.* **26,** 79–83.
26. Pelham, H. R. B. (2000) *Methods Enzymol.* **327,** 279–283.

APPENDIX III

Example 17: Genus-species with widely varying species

Specification: The specification discloses the rat cDNA sequences for proinsulin and pre-proinsulin and a method for determining the corresponding human and other mammalian insulin cDNA sequences. However, the specification does not disclose any actual cDNA sequence other than the rat proinsulin and pre-proinsulin sequence. The specification discloses that one human proinsulin amino acid (but not cDNA) sequence was known at the time of filing. The art recognized that the sequence of human insulin proteins, and therefore also cDNAs, would probably vary among individuals. The specification also discloses that pre-proinsulin is post translationally modified to form proinsulin, and that proinsulin is cleaved to form insulin.

Claims:

Claim 1. An isolated mammalian cDNA encoding insulin.

Claim 2. The isolated cDNA of claim 1 wherein the mammalian cDNA is human.

Analysis: The examiner should analyze claim 2 first because it is drawn to a subgenus of the genus of claim 1.

Claim 2:

A review of the full content of the specification indicates that human cDNA molecules that encode insulin are essential to the operation/function of the invention.

Claim 2 is directed to a genus of human cDNA which encodes insulin.

There is no species of human insulin cDNA disclosed.

Based upon art published after applicant's filing date there is expected to be variation among the species of cDNA which encode human insulin because the sequence of human insulin proteins, and therefore also human insulin cDNAs, would be expected to vary among individuals.

The specification discloses only the sequence of a single human proinsulin protein, and does not disclose any human cDNA sequence at all.

In addition, there is no evidence on the record of a relationship between the structure of rat insulin cDNA and the structure of insulin cDNAs from humans or other mammals that would provide any reliable information about the structure of other insulin cDNAs on the basis of the rat insulin cDNA.

There is no evidence on the record that the disclosed rat cDNA proinsulin sequence had a known structural relationship to the human cDNA sequence, or to other mammalian cDNA sequences; the specification discloses only a single human proinsulin (protein) sequence; the art indicated that human proinsulin proteins were expected to be variable in structure; and there is expected to be variation among human cDNAs that

encode a given human proinsulin. In view of the these considerations, a person of skill in the art would not have viewed the teachings of the specification as sufficient to show that the applicant was in possession of the claimed human cDNA.

Claim 1:

Claim 1 is directed to a genus of mammalian cDNAs which encode insulin. The specification evidences actual reduction to practice of the rat cDNA sequences for proinsulin and preproinsulin, but does not disclose any other cDNA sequences. The art indicates that there is likely to be substantial variation among the species within the genus of cDNAs that encode mammalian insulins because the sequences of the mammalian insulin proteins, and therefore the mammalian cDNAs, would be expected to vary among species.

The specification discloses a method for determining the corresponding human and other mammalian insulin cDNA sequences as well as the function of the claimed sequences. However, neither the specification nor the general knowledge of those skilled in the art provide evidence of any partial structure which would be expected to be common to the members of the genus. Moreover, there is post filing date evidence that indicates that there is a lack of a structural relationship between the rat insulin cDNA sequences and other mammalian insulin cDNA sequences. In view of the above considerations one of skill in the art would not recognize that applicant was in possession of the necessary common features or attributes possessed by members of the genus, because rat cDNA sequences are not representative of the claimed genus. Consequently, since applicant was in

possession only of the rat insulin cDNA and since the art recognized variation among the species of the genus of cDNAs that encode mammalian insulin, the rat insulin cDNA was not representative of the claimed genus. Therefore, the applicant was not in possession of the genus of mammalian insulin cDNAs as encompassed by claim 1.

Conclusion:

Claims 1 and 2 do not meet the written description requirement.

This Page is Inserted by IFW Indexing and Scanning Operations and is not part of the Official Record

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ BLACK BORDERS
- ☑ IMAGE CUT OFF AT TOP, BOTTOM OR SIDES
- ☐ FADED TEXT OR DRAWING
- ☑ BLURRED OR ILLEGIBLE TEXT OR DRAWING
- ☐ SKEWED/SLANTED IMAGES
- ☐ COLOR OR BLACK AND WHITE PHOTOGRAPHS
- ☐ GRAY SCALE DOCUMENTS
- ☐ LINES OR MARKS ON ORIGINAL DOCUMENT
- ☐ REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY
- ☐ OTHER: _____

IMAGES ARE BEST AVAILABLE COPY.
As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.